

Effects of dietary nutrient composition on de novo lipogenesis in gilthead sea bream (*Sparus aurata*)

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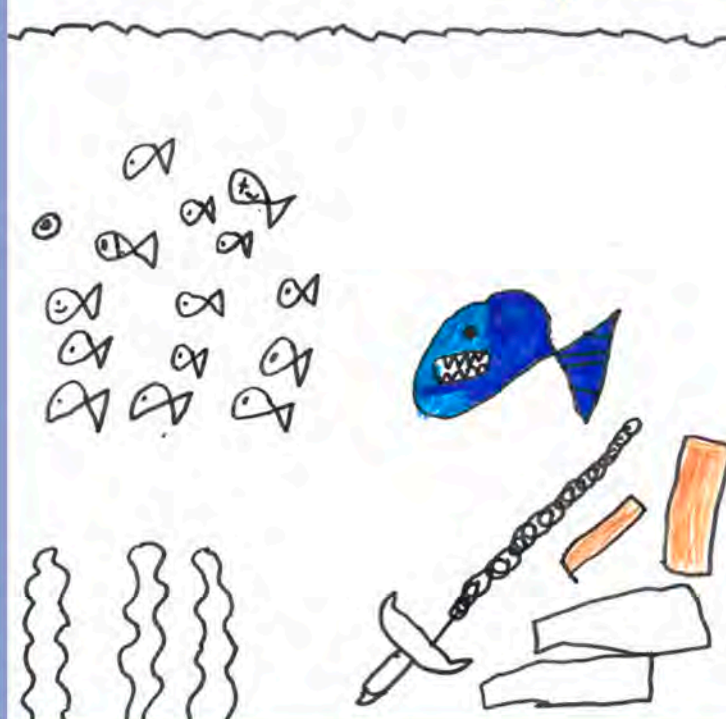
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Effects of dietary nutrient composition on *de novo* lipogenesis in Gilthead sea bream (*Sparus aurata*)

PhD Thesis



Written by Kim Schøn Ekmann
Defended 17 January 2013

**Effects of dietary nutrient composition on *de novo* lipogenesis in Gilthead sea bream
(*Sparus aurata*)**

Ph.D. thesis by

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October 2012

Technical University of Denmark

National Institute of Aquatic Resources

Section for Aquaculture

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Colophon

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Preface

In the early days of March 1993 a wee lad was knocking on the door to a young aquaculture institute called Danish Institute for Fisheries Technology and Aquaculture (DIFTA) at the North Sea Centre asking for a job. He was sincerely interested in biology in general and aquaculture in particular, and after a less-than-impressive upper secondary school graduation he needed to earn credit in the form of relevant student work to get accepted to the biology study at Aarhus University. After six months of interesting work nurturing gilthead sea bream fry amongst other things the wee lad was accepted into University. During four years of studies the lad was introduced to many interesting fields of biology, but aquaculture seemed to be the recurring topic of interest. This lead him back to DIFTA late 1998 to do his Master's thesis on utilisation of dietary lipid in gilthead sea bream, which became part of a collaboration project between DIFTA, BioMar and Research Centre Foulum. Soon after obtaining his M.Sc. degree he was employed as product developer at BioMar's biological trial station in Hirtshals, Denmark, and after almost ten years of employment he was offered to pursue a Ph.D. degree as cooperation between BioMar and Technical University of Denmark. That (lucky) lad was me ☺

During the last part of this journey I have been truly blessed with the help and patience of many people around me. Therefore I would like to thank my supervisors Associate professor Dr. Peter V. Skov and Dr. Anne Johanne T. Dalsgaard, both from Technical University of Denmark, Section for Aquaculture and Dr. Patrick J. Campbell and M.Sc. Jørgen Holm from BioMar. Also, I would like to thank my colleagues at BioMar for fruitful late-night discussions about fish nutrition and more, and to my colleagues and fellow students at DTU Aqua. In addition a lot of technical staff at BioMar's trial station, Risø DTU and DTU Aqua has been of invaluable help during my study. In particular I would like to thank Ulla, Dorte and Brian at DTU Aqua's lab for all their help and patience with me. Additionally, I would like to thank the guys at BioMar's trial station for all their help during trialling: Per E., Per S., Jon, Ly, Bo and Kim. Also, thank you Marine for taking some of my late night shifts when trialling fish. Finally, my sincerest gratitude goes to Stinne and the boys. Thank you for putting up with me through this - I could not have done it without you.

Hirtshals, October 31st 2012



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List of papers

- Paper I: Ekmann, K.S.; Dalsgaard, J.; Holm, J.; Campbell, P.J.; Skov, P.V., 2012. Glycogenesis and *de novo* lipid synthesis from dietary starch in juvenile gilthead sea bream (*Sparus aurata*) quantified with stable isotopes. British Journal of Nutrition, in press
- Paper II: Ekmann, K.S.; Dalsgaard, J.; Holm, J.; Campbell, P.J.; Skov, P.V., 2012. *De novo* lipid synthesis from dietary protein in gilthead sea bream (*Sparus aurata*) quantified with stable isotopes - effects of DP/DE ratio. Submitted to British Journal of Nutrition.
- Paper III: Ekmann, K.S.; Dalsgaard, J.; Holm, J.; Campbell, P.J.; Lund, I.; Skov, P.V., 2012. Effects of substituting lipid with starch as dietary energy source on performance, nutrient utilization and fatty acid composition in gilthead sea bream (*Sparus aurata*) juveniles. Submitted to Comparative Biochemistry and Physiology, Part A.

List of abbreviations and biochemical terms

AA:	Amino acid
Acetyl-CoA:	Acetyl coenzyme A. Key metabolic precursor.
aCLR:	Apparent crude lipid retention (in % of crude lipid intake)
ADC:	Apparent digestibility coefficient. The percentage of an ingested nutrient not recovered in the faeces.
aDLR:	Apparent digestible lipid retention (in % of digestible lipid intake)
APE:	Atom percent excess. A measure of the abundance of a stable nuclide in a sample expressed in terms of the excess, in atom percent, over that naturally present. It is used to express the extent of enrichment or dilution of substances labelled with stable isotopes.
ARA:	Arachidonic acid. A poly unsaturated n-6 fatty acid (20:4n-6)
β -oxidation:	β -oxidation is the process by which fatty acids, in the form of acyl-CoA molecules, are broken down to generate acetyl-CoA
CPR:	Crude protein retention (in % of crude protein intake)
DE:	Digestible energy
DER:	Digestible energy retention (in % of digestible energy intake)
DHA:	Docosahexaenoic acid. A poly unsaturated n-3 fatty acid (22:6n-3)
DP/DE ratio:	Ratio between dietary DP (g/kg) and DE (MJ/kg)
DP:	Digestible protein
DPA:	Docosapentaenoic acid. A poly unsaturated n-3 fatty acid (22:5n-3)
DPR:	Digestible protein retention (in % of digestible protien intake)
EA:	Elemental analyzer
EAA (IAA):	Essential/indispensable amino acid
EFA:	Essential fatty acid
EPA:	Eicosapentaenoic acid. A poly unsaturated n-3 fatty acid (20:5n-3)
FA:	Fatty acid
FADH ₂ :	The reduced version of flavin adenine dinucleotide (FAD). Used as electron carrier used for oxidative phosphorylation
FAS:	Fatty acid synthase. A multi-enzyme protein which catalyses fatty acid synthesis
Fatty acyl desaturases:	Enzyme which removes two hydrogen atoms from a fatty acid, creating a carbon-carbon double bond

FbPase:	Fructose biphosphatase. An enzyme that converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis
FCR:	Feed conversion ratio (feed used / biomass gain)
FI:	Feed intake
G6P:	Glucose 6-phosphate. Product of the phosphorylation of glucose prior to glycogenesis or glycolysis
G6PD/G6PDH:	Glucose-6-phosphate dehydrogenase (G6PD or G6PDH). An enzyme of the pentose phosphate pathway which supplies reducing energy to cells (NADPH)
Gluconeogenesis:	A metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids
Glycogenesis:	Glycogen synthesis
Glycolysis:	The metabolic pathway that converts glucose into pyruvate
HSI:	Hepatosomatic index (liver weight / total body weight)
IAA (EAA):	Indispensable/essential amino acid
IRMS:	Isotope ratio mass spectrometry. A method which allows for the determination of abundance and isotopic composition of a certain compound
Ketogenesis:	Production of ketone bodies (e.g. acetone or acetoacetic acid)
LDPO:	The percentage of the lipiddeposition which can be attributed to digested protein (<i>via</i> lipogenesis)
LDR:	Deposition rate of lipid derived from digested starch (<i>via</i> lipogenesis)
Lipogenesis:	The process by which acetyl-CoA is converted into fats
Malonyl-CoA:	Malonyl coenzyme A
MOPA:	Proximate chemical analysis of Moisture, Oil, Protein and Ash
MUFA:	Mono unsaturated fatty acid
NADPH:	The reduced form of nicotinamide adenine dinucleotide phosphate (NADP+) which provides the reducing equivalents for biosynthetic reactions (such as lipogenesis)
PFK:	Phosphofructokinase is a kinase enzyme that phosphorylates fructose 6-phosphate in glycolysis
PUFA:	Poly unsaturated fatty acid
Pyruvate:	The metabolic product of glycolysis (conversion of glucose to pyruvate)
RPCL:	Recovery of protein carbon in whole fish lipid fraction (%)

RSCL:	Recovery of starch carbon in whole fish lipid fraction (%)
SAFA:	Saturated fatty acid
SGR:	Specific growth rate
TCA cycle:	The tricarboxylic acid cycle = the Krebs cycle = the citric acid cycle
VSI:	Viscerosomatic index (visceral weight / total body weight)

Dansk resumé (abstract in Danish)

På trods af mere end 20 års forskning og intensiv akvakultur har guldbrasen fortsat en væsentlig ringere foderudnyttelse end mange andre arter i akvakultur. Hvor laksefisk i opdræt (ved slagtestørrelse) typisk kræver mellem 0,9 og 1,1 kg foder pr. kg tilvækst, kræver guldbrasen typisk 1,6 til 2,0 kg, hvilket også fremgår af den effektivitet hvormed protein tilbageholdes som vækst i fisken. Mens laksefisk tilbageholder helt op til 55% af den foderprotein mængde som de æder, tilbageholder guldbrasen typisk mindre end 30%. Indtil videre er der intet der tyder på, at forskelle i næringsstoffordøjeligheden kan forklare disse forskelle, da guldbrasen i store træk fordøjer næringsstoffer med samme eller højere effektivitet end laksefisk.

Da fordøjede næringsstoffer endogent kan omdannes til andre næringsstoffer eller metabolitter i fisken, kan det være svært kvantitativt at konkludere noget om deres skæbne. Brugen af stabile isotoper (så som ^{13}C mærket stivelse eller protein) som biokemiske markører giver mulighed for, at spore individuelle næringsstoffer og bestemme i hvilket omfang de omdannes til andre metabolitter endogent. Denne afhandling indeholder 3 artikler der belyser den endogene omdannelse af foder stivelse og protein til kropsfedt i fisken, samt konsekvensen heraf på fiskens fedtsyre profil.

Resultaterne fra artikel I viste, at mellem 4,2 og 8,4% af den fordøjede stivelse blev omdannet til kropsfedt endogent, svarende til en syntese hastighed på 18,7 til 123,7 mg/kg biomasse/dag, når fiskene åd iso-protein og iso-energi diæter med et stivelsesindhold på henholdsvis 6 og 24%. Desuden kunne op til 68,8% af lever glykogen indholdet tilskrives stivelse, mens det samme gjorde sig gældende for 38,8% af glykogen indholdet i hel fisk. Omvendt betød dette, at op mod 2/3 af hel-krops glykogenet, samt omtrent 1/3 af lever glykogenet kunne tilskrives andre kilder end stivelse, selv med et højt foderstivelses niveau.

Ved brug af 9 diæter med varierende fordøjeligt protein (DP) indhold (33 – 40%) og fordøjeligt energi (DE) indhold (19,5 – 21,5 MJ/kg) viste resultaterne fra artikel II, at mellem 18,6 og 22,4% af det fordøjede protein blev konverteret til kropsfedt endogent, svarende til mellem 21,6 og 30,3% af den totale kropsfedt deponering under forsøget. Tilbageholdelseeffektiviteten af fodernæringsstoffer samlet, viste en stigende tilbageholdelse af protein når foderets DP/DE forhold faldt, mens det modsatte var tilfældet for fedt, hvilket tyder på, at deamineret DP faktisk blev omdannet til fedt endogent. Der blev i tillæg observeret en meget tydelig forbedring af foderkvotienten (FCR) med stigende DE indhold i foderet, hvilket i kombination af en forbedret protein tilbageholdelsesevne med faldende

DP/DE forhold indikerer at guldbrasen er i stand til effektivt at udnytte diæter over et stort spænd af DP/DE forhold og energi tætheder.

Resultaterne fra artikel III viste, at både den tilsyneladende tilbageholdelse af fedtsyrer, samt den resulterende hel-krops fedtsyre profil i fisken begge var tydeligt påvirket af foderets stivelsesindhold (varierende fra 6 til 24% i iso-DP og iso-DE diæter). Den tilsyneladende tilbageholdelse af mættede fedtsyrer (SAFA) og monoumættede fedtsyrer (MUFA) var direkte korreleret med foderets stivelsesniveau (og omvendt korreleret med foderets fedtindhold), og oversteg 100% i fisk som fik høj-stivelses diæter, hvilket indikerer endogen fedtsyntese i disse fisk. Omvendt var den tilsyneladende tilbageholdelse af flerumættede fedtsyrer (PUFA) upåvirket af foderets stivelsesindhold. Kombineret forårsagede disse resultater et forhøjet SAFA og MUFA indhold, samt reduceret PUFA indhold i hel fisk, når disse blev fodret med stigende mængder stivelse.

Når resultaterne fra alle 3 artikler ses samlet, synes guldbrasen at bestræbe sig på, at opretholde en bestemt kropsenergi status over en bred vifte af DP/DE niveauer, energitætheder og næringsstofsammensætninger i foderet, også selv om store mængder protein ofres for at opnå dette. Dette kan tyde på, at denne art har udviklet sig til at maksimere energilagring, som kan anvendes til migrerings- eller reproduktionsformål, frem for en mere effektiv udnyttelse af protein til vækst. Endogen fedtsyntese synes at spille en vigtig rolle i denne energihomeostase.

Abstract

Despite more than 20 years of nutritional research and intensive culture, gilthead sea bream appear to utilise diets inherently worse than many other species in aquaculture. Thus, while salmonids at typical slaughter size typically require between 0.9 – 1.1 kg of feed for growing one kg, gilthead sea bream typically require between 1.6 – 2.0 kg, which is similarly reflected in the efficiency with which dietary protein is retained in body growth. While salmonids have been reported to retain as much as 55% of the dietary protein as growth, gilthead sea bream typically retains less than 30%. So far, there are no indications that differences in nutrient digestibility coefficients can explain these differences, since gilthead sea bream largely digests dietary nutrients similarly or better than salmonids.

As dietary nutrients upon digestion can be endogenously converted into other nutrients or metabolites, it can be hard to quantitatively conclude on the fate of them. Using stable isotope tracers (such as ^{13}C labelled starch or protein) allows us to trace specific nutrients and determine to which extent they are endogenously converted into other metabolites.

The present thesis comprises three supporting papers which look into the conversion of dietary starch and protein into body lipids as well as the consequences of this on fatty acid profile of the fish.

Results from paper I showed that between 4.2 and 8.4% of digested starch was converted into body lipids *de novo*, corresponding to a synthesis rate of 18.7 to 123.7 mg/kg biomass/day, when feeding iso-DP and iso-DE diets ranging between 6 and 24% dietary starch, respectively. Additionally, up to 68.8% of the hepatic glycogen pool could be attributed to dietary starch, while the same was true for up to 38.8% of the whole body glycogen pool. In turn, this implies that almost two thirds of the whole body glycogen and approximately one third of the liver glycogen must have originated from sources other than dietary starch, even when feeding the high starch diet.

Using nine experimental diets differing in dietary DP (33 – 40%) and DE (19.5 – 21.5 MJ/kg), results from paper II showed that between 18.6 and 22.4% of the DP was converted into lipid *de novo*, corresponding to between 21.6 and 30.3% of the total lipid deposited in the fish during the study. The nutrient retention results combined showed that while protein was spared by a decreasing dietary DP/DE level, the opposite was true for lipid, substantiating that deaminated DP was indeed converted into body lipids. Additionally, a very clear improvement of FCR with increasing DE level combined with an improvement of digestible protein retention with decreasing DP/DE levels suggest that gilthead sea bream are

capable of efficiently utilising feeds within a wide range of dietary DP/DE ratios and energy densities.

Results from paper III showed that both fatty acid retention dynamics and final fatty acid profile of the fish were clearly influenced by an increment in dietary starch content (using diets otherwise iso-DP and iso-DE). The apparent retention of saturated fatty acids (SAFA) and mono unsaturated fatty acids (MUFA) were positively related to dietary starch level (and negatively related to dietary lipid level), exceeding 100% in fish fed high starch diets. These findings substantiate that considerable *de novo* lipogenesis was taking place and apparently subject to nutritional control, while apparent retention of poly unsaturated fatty acids (PUFA) appeared to be un-affected by dietary treatment. Combined, this caused the SAFA and MUFA content of the fish to increase and the PUFA content to decrease when increasing dietary starch level, adversely affecting the overall FA quality of the final product. Considering lipogenesis results, nutrient retention efficiencies and body composition results obtained in the three trials collectively, gilthead sea bream appear to endeavour to rigorously maintain a certain whole body energy status under a wide variety of dietary DP/DE ratios, energy densities and nutrient compositions, even if substantial amounts of dietary protein is sacrificed to achieve this. This may indicate that this species has evolved to maximise energy storage in the form of lipid for seasonal, migratory or maturation purposes at the expense of increasing body size through more efficient use of protein for growth. *De novo* lipogenesis appear to play a key role in maintaining this energy homeostasis.

1. Introduction and background

Aquaculture is the fastest growing food producing industry in the world, and almost 50% of the fish used for human consumption is now of aquaculture origin. The total, global aquaculture production has exceeded 80 million tonnes, having had an average annual growth rate of 6.6% over the last 40 years⁽¹⁾. Fish meal and fish oil from wild fisheries are major constituents of fish feeds, and the growth in aquaculture has exerted a huge demand for these commodities⁽²⁾, which has coincided with decreasing catches. Combined, this has caused fish meal and fish oil prices to triple within the last decade, which in turn has increased prices on compound fish feeds dramatically. As a consequence, a substantial number of studies have been undertaken to find suitable fish meal and fish oil replacements, including protein from vegetable raw materials⁽³⁾, animal by-products^(4, 5) or single-cell organisms⁽⁶⁻⁸⁾, and lipids of plant origin^(9, 10). Macronutrients in the natural diet of carnivorous fish comprise mainly protein and lipids abundant in essential amino acids and fatty acids, respectively, while they are basically free of anti-nutrients.

The introduction of vegetable raw materials into aquaculture feeds has caused a concomitant increase in dietary carbohydrate, challenging the carnivorous nature of the digestive and oxidative processes associated with metabolism in many cultured species^(11, 12). Carnivorous fish, including gilthead sea bream (*Sparus aurata*) do, however, appear to possess the digestive and metabolic enzymatic apparatus needed to utilize digestible carbohydrates^(11, 13). A maximum digestible carbohydrate inclusion level of 20% has been recommended for marine fish and salmonids, while up to 40% may be included in diets for warm water omnivorous species^(12, 14). Enes *et al.*⁽¹³⁾ recommended a maximum inclusion level of 20% digestible carbohydrate in diets for juvenile gilthead sea bream and European sea bass (*Dicentrarchus labrax*).

Regardless of raw material source, the main concern in feed production is the quality, quantity and ratio between the nutrients brought by these. However, when optimising diets for farmed animals including fish it must be recognised that the nutritional requirements of a species are not absolute. Rather, dietary nutrients should be present in the correct proportion to each other as pointed out by Wilson⁽¹⁵⁾. Consequently, recommendations on major nutrients are typically given as the ratio between digestible protein (DP) and digestible energy (DE), where the optimal DP/DE ratio is the minimum amount of DP required for optimising a certain production trait (e.g. feed utilisation, growth or nutrient retention efficiency). Diets with DP/DE ratios higher than optimal contain more DP than can be

accreted with the amount of DE available, which causes excessive protein catabolism, which in turn increases the amount of nitrogenous compounds discharged into the environment^(16, 17). While any of the three macro nutrients (protein, lipids and carbohydrates) may be in excess in a diet, only lipid (in the form triglycerides) can be stored in substantial amounts in fish. As a consequence, fish (and other animals) have evolved metabolic pathways through which energy (and carbon) from either of the major energy yielding nutrients can be transformed into lipids *via* what could be considered a metabolic junction molecule, namely acetyl-CoA. This process is termed *de novo* lipogenesis. While there is agreement that acetyl-CoA is the primary carbon donor in lipogenesis, and that the process is fuelled by the reducing powers of NADPH, there is much more discussion on the extent to which carbohydrates (glucose) and proteins (amino acids) precede acetyl-CoA for lipogenic purposes, and which biochemical processes and regulatory mechanisms are responsible in this process.

In gilthead sea bream several studies have assessed the regulatory effects of dietary carbohydrate or protein level on activity of liver enzymes involved in glycolysis, gluconeogenesis, glycogenesis, lipogenesis and amino acid catabolism⁽¹⁸⁻²⁷⁾. However, since protein and carbohydrates share some common parts of their degradative pathways, and thus are able to precede identical metabolites such as pyruvate, acetyl-CoA, lipids or glycogen (*via* gluconeogenesis/glycogenesis) it is difficult to quantitatively conclude on the metabolic fate of these nutrients. Similarly, studies on the key enzymes of carbohydrate and protein metabolism typically examine enzyme activity as an indicator of metabolite fluxes, but does not quantify end products or determine their origin.

The technique of using stable isotopes in nutritional studies dates back almost 80 years⁽²⁸⁾ and makes it possible to trace the fate of various compounds in the animal body, assuming that the tracer behaves functionally similar to the tracee but still can be distinguished from the tracee by measurement. In fish, isotope labelled macro nutrients have been previously administered to Atlantic cod (*Gadus morhua*)⁽²⁹⁾, rainbow trout (*Oncorhynchus mykiss*)⁽³⁰⁻³⁴⁾, Atlantic halibut (*Hippoglossus hippoglossus*)⁽³⁵⁾, Atlantic salmon (*Salmo salar*)^(36, 37), European flounder (*Pleuronectes flesus*)⁽³⁸⁾ and gilthead sea bream⁽³³⁾ as a single oral bolus or administered intraperitoneally, allowing researchers to determine the metabolic fate of nutrients and estimate synthesis rates of their derived metabolites.

The objectives of the research behind the present thesis were to quantify *de novo* lipogenesis in gilthead sea bream when: 1) changing the main dietary energy source from lipid to starch (paper I), and 2) varying the dietary DP:DE ratio and energy density (paper II) by applying

^{13}C labelled nutrients (starch and protein) as metabolic tracers. Also, the effects of fatty acids synthesised *de novo* on resulting whole body fatty acid profile and accretion of fatty acids were assessed (paper III). Throughout both the present thesis and supporting papers the terms ‘apparent lipid retention’ or ‘apparent fatty acid retention’ have been used. As deposited lipid/FAs could originate from both dietary and endogenous sources, it would be erroneous to use the term ‘retention’. Thus, all lipid/FA retention efficiencies are henceforth referred to as “apparent”.

The present thesis comprises a brief introduction to general gilthead sea bream biology and aquaculture history followed by a general introduction of the three major dietary nutrients (proteins, lipids and carbohydrates) and their metabolic pathways and fates in fish. Chapter 1.4 describes the biochemical background of *de novo* lipogenesis, while chapter 1.5 describes stable isotopes and their use in nutritional studies. The most important results are recapitulated in chapter 5, while conclusions and future perspectives of the present work are found in chapter 6 and 7, respectively.

1.1 Gilthead sea bream biology

Gilthead sea bream (*Sparus aurata*) is geographically distributed around the rim of the Mediterranean Sea, along the Eastern Atlantic coast from Senegal to the British Isles, and has also been reported more scarcely in the North Sea and the Black Sea (figure 1).

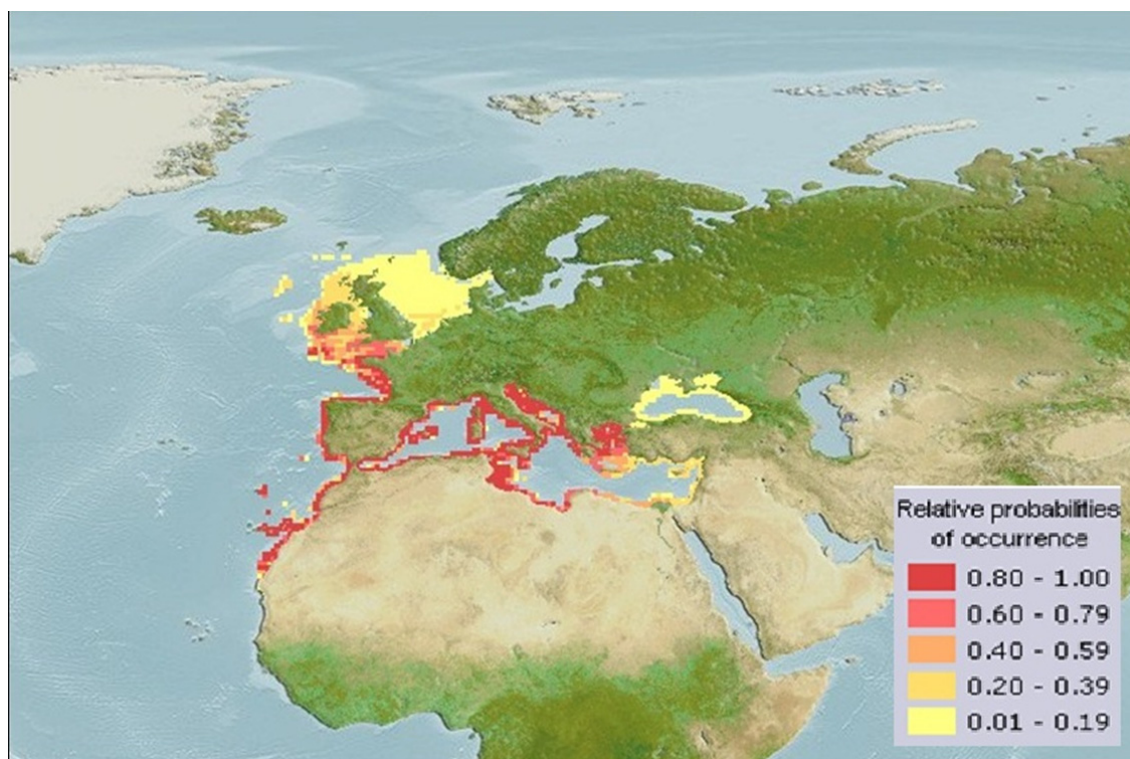


Figure 1. Reviewed Native Distribution Map for *Sparus aurata* (Gilthead seabream). www.aquamaps.org, version of Aug. 2010. Web. Accessed 18 Sep. 2012.

Gilthead sea bream is a demersal species living at depths between 0 to 30 meters on rocky and sandy grounds as well as near sea grass beds, but can occasionally be found on depths of 150 meters. They live either solitary or in small aggregations, and younger fish are often found in protected coastal lagoons and estuaries in the spring. In the late autumn adult fish return to the open sea to breed. The species is protandrous hermaphrodite, meaning that it starts life as a male, but changes sex at a later life-stage. In the wild, males sexually mature at two years of age (20-30 cm), while females mature at two-three years of age (33-40 cm). In farming conditions the timing of the sex reversal will depend on nutritional, social and hormonal factors. The natural diet of gilthead sea bream is mainly crustaceans and shellfish, which is why the powerful jaws, are equipped with both molars and canines⁽³⁹⁾.

1.2 Gilthead sea bream in aquaculture

1.2.1 History of Mediterranean aquaculture

Aquaculture in the Mediterranean area dates back to the Etruscan civilization (approximately 600 B.C.), the purpose at the time being more related to storage of high quality foods than actual farming. Virtually all aquaculture collapsed along with the fall of the Roman Empire and was not revived again before the 12th to 18th century. Again, at that time storage was the main reason for keeping fish in culture. This was due to the Catholic practice of abstaining from meat on Fridays, causing a demand for fresh fish on that particular weekday greater than could be supplied by fishermen. Thus, keeping fish in captivity was a practical way of levelling out demand over time. In the 19th century farming of shellfish became common, particularly in the western Mediterranean and the Adriatic Sea.

1.2.2 Culture practice and development

Up until the beginning of the 1980's gilthead sea bream was mainly cultured in coastal lagoons and saltwater ponds where low-density rearing systems served to trap fish, taking advantage of the natural migratory patterns of juveniles from the sea into lagoons. Artificial breeding was successfully achieved in Italy in 1981-82 and large-scale production of juveniles was achieved in Spain, Italy and Greece in 1988-89⁽¹⁾. This definitively closed the life-cycle of gilthead sea bream in captivity, and triggered an explosive development in commercial farming up until now (figure 2). As a consequence of the increasing availability of farmed gilthead sea bream on the market, prices have since decreased by approximately 60%, making it difficult for farmers to make a profit. Today, Greece is the biggest gilthead sea bream farming nation by far, supplying 49% of the global commercial production, followed by Turkey (15%), Spain (14%), and Italy (6%). Other producing countries include Croatia, Cyprus, Egypt, France, Malta, Morocco, Portugal and Tunisia. On a more general note the overall development in global aquaculture has shown even more impressive yearly growth rates. Today, the global aquaculture production (including fish, crustaceans, molluscs and aquatic plants) exceeds 80 million tonnes annually (figure 3), which means that gilthead sea bream account for less than 0.2% of the global aquaculture production today⁽¹⁾.

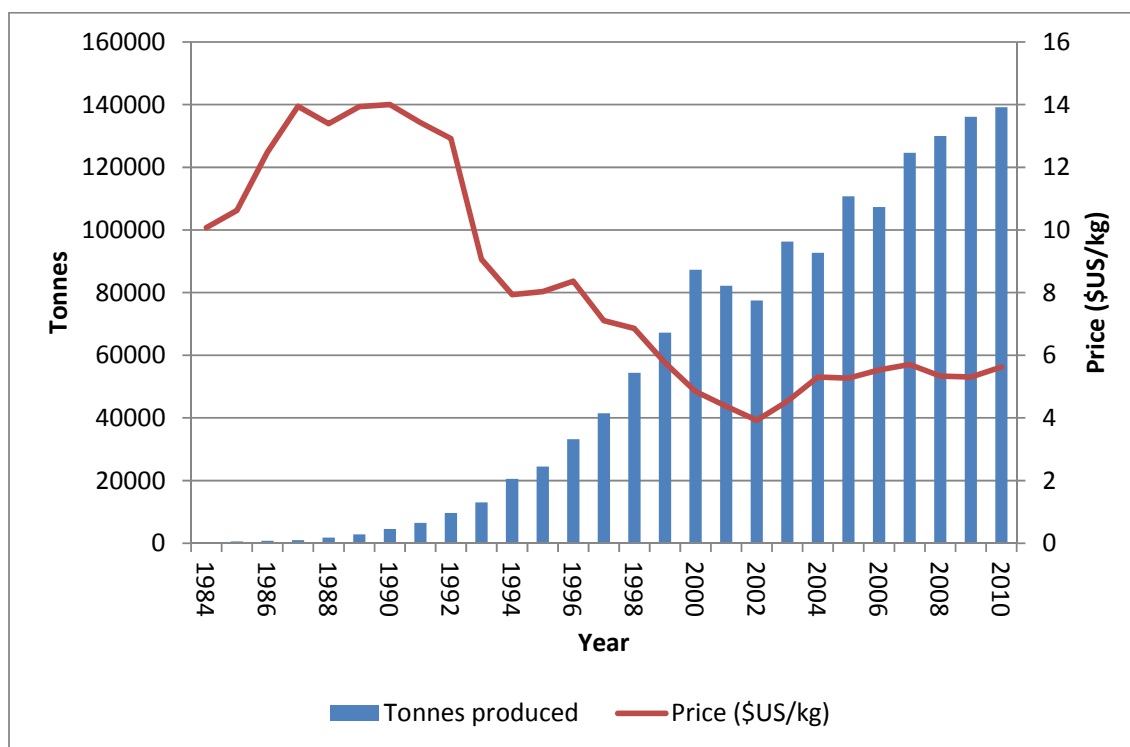


Figure 2. Yearly production of gilthead sea bream and development in fish price, 1984-2010, as reported by the FAO⁽¹⁾.

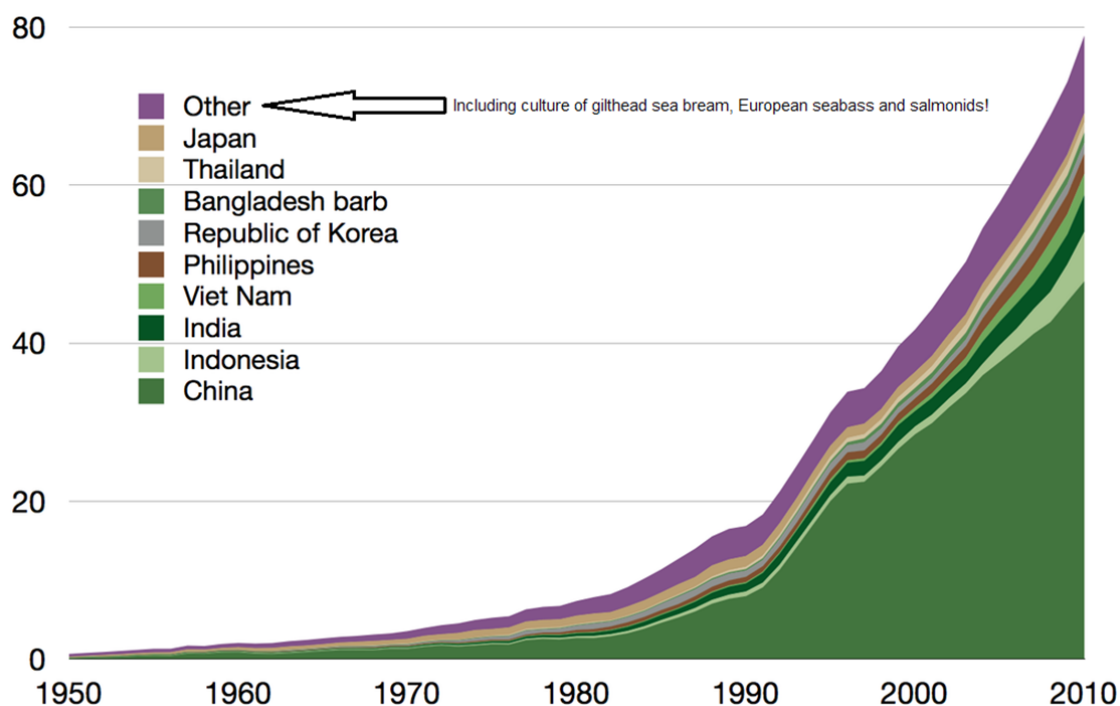


Figure 3. Global aquaculture production in million tonnes, 1950–2010, as reported by the FAO⁽¹⁾.

1.3 Nutritional requirements

1.3.1 General introduction

Fish, like other living organisms, require nutrients to sustain life processes, growth, activity and reproduction. The three major energy yielding nutrients are proteins, lipids and carbohydrates which all serve as precursors for biosynthesis of structural and storage molecules, enzymes or intermediate metabolites. Also, all of them potentially serve as sources of free energy needed for maintaining life processes. The extent to which each of the major nutrients are directed towards anabolic, catabolic or intermediary metabolic purposes is determined by a number of biotic (overall energy status of the fish, species (genetics), life-stage, stress and health status) and abiotic factors (temperature, oxygen availability, salinity and photoperiod). In addition to the three major nutrients a number of organic (vitamins) and inorganic (minerals) micronutrients are vital for normal function and growth of fish. These will not be considered in further detail in the present thesis, and in the following studies they are not considered to be limiting or inhibitory to normal function and growth of fish. In the following sub-sections of the present section (1.3.2 – 1.3.4), the three major nutrients, their uptake, functions and biochemical pathways will be briefly introduced, followed by a brief review of the nutritional regulation and requirements of gilthead sea bream. The fate of yielded metabolic precursors needed for *de novo* lipogenesis, acetyl-CoA and NADPH, is in this section only mentioned peripherally, but will be discussed further in detail in section 1.4.

1.3.2 Proteins

Proteins constitute the biggest pool of organic matter in fish (~50-75% on dry weight basis) and play a very important role in both structure and metabolism of all living organisms. Proteins comprise chains of amino acids held together by peptide bonds, and contain, as the only of the macro nutrients, both nitrogen and sulphur. In biological material protein is comprised by 20 different AAs of which 10 are considered to be essential, meaning that they cannot be biosynthesised within the fish itself, thus have to be supplied through the diet^(15, 40). The remaining 10 non-essential AAs can be synthesised endogenously from one of four common metabolic intermediates: pyruvate, oxaloacetate, α -ketoglutarate, or 3-phosphoglycerate⁽⁴¹⁾. Upon ingestion proteins are degraded through a cascade of processes, including acidic denaturation (pH 3-5) and pepsin degradation in the stomach of the fish, followed by an alkaline (pH 8.5-9.5) enzymatic degradation (trypsin, chymotrypsin, carboxypeptidase etc.) yielding a blend of free AAs, di- and tripeptides which are all capable

of crossing the intestinal wall. These nutrients are mainly taken up in the mid intestine of the fish *via* either passive or active transport (requiring Na^+ ions). The absorption of peptides / amino acids from the intestinal tract is usually very efficient in fish, and typically only 5-15% of these nutrients are lost *via* faeces⁽⁴²⁾. Amino acids obtained from the diet are used for multiple purposes such as body protein synthesis, synthesis of biomolecules (porphyrines, purines, pyrimidines, neurotransmitters, hormones, complex lipids and amino sugars), or they can undergo deamination yielding an amino group, which is excreted as ammonium or transferred to another α -keto acid, producing another (non-essential) amino acid in a process termed transamination (figure 4). The residual α -keto acid may subsequently be converted into one of six intermediates of the TCA cycle (depending on the nature of the α -keto acid in question): α -ketoglutarate, succinyl-CoA, fumarate, oxaloacetate, pyruvate or acetyl-CoA, which in turn may be utilised for *de novo* lipogenesis, gluconeogenesis, yield ketone bodies, or be completely oxidised for energy purposes (figure 5).

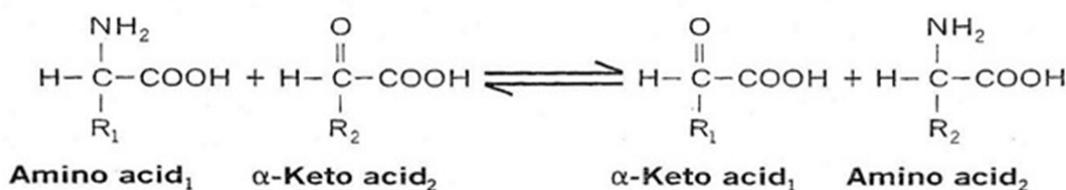


Figure 4. The transamination process

The metabolic fate of dietary amino acids should not be considered a strict one-way process. Rather, proteins are continuously synthesised and degraded, meaning that amino acids are also in a continuous state of flux in the animal body⁽⁴³⁾. The balance between protein synthesis and protein degradation is termed protein turnover. Thus, if protein degradation exceeds protein synthesis there is a net loss of protein, while a net protein gain is the result of protein synthesis exceeding protein degradation⁽⁴⁴⁾. Protein synthesis is a complex process where DNA is transcribed to produce a specific mRNA template, which in combination with a tRNA/ribosome complex fuse amino acids in a specific sequence to make a polypeptide chain, which upon completion comprise the target protein. Due to the rigid amino acid demands of these protein ‘blueprints’, amino acids should be supplied in the correct proportion to support protein synthesis. This is particularly true for the essential amino acids which cannot be biosynthesised *de novo*. Since fish, like other animals, have only very limited means of storing excess amino acids, the efficiency with which dietary amino

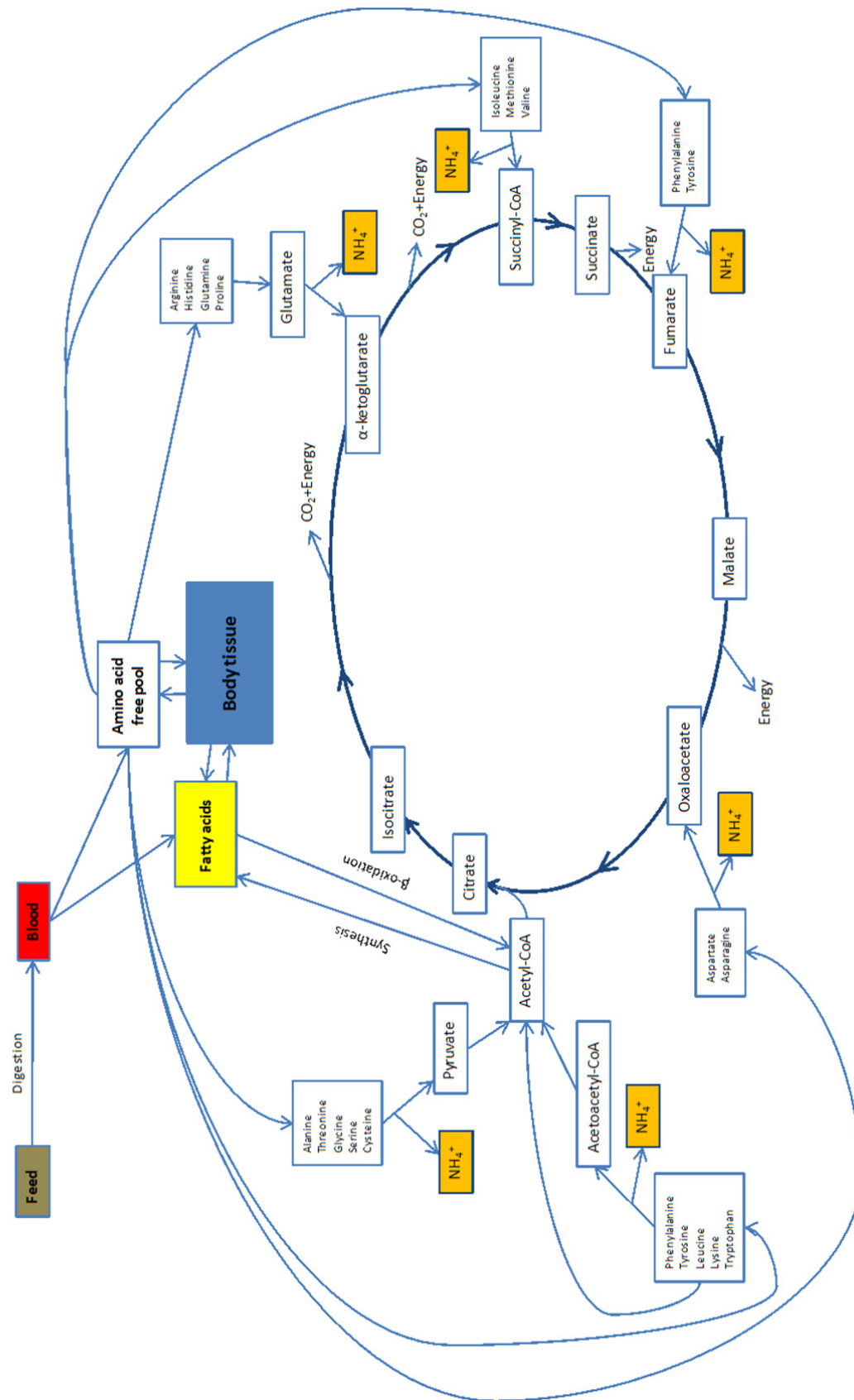


Figure 5. Entrance of amino acids into the TCA cycle and simplified nutrient flows.

acids are assimilated to biomass largely depend on the availability of the essential amino acid which will be exhausted first ('first limiting EAA'), and the amount of metabolic energy available to support growth. Thus, the efficiency with which dietary protein is retained as growth not only depends on the nature of the protein itself. Rather, the efficiency is highly influenced by the availability of energy originating from nutrients other than protein. Therefore, it makes more sense to express protein requirements relatively to dietary energy content, or as expressed by Wilson (2002)⁽¹⁵⁾: *"Fish, like other animals, do not have a true protein requirement but have a requirement for a well-balanced mixture of essential or indispensable and nonessential or dispensable amino acids...The optimal dietary protein level for fish, as well as other animals, is influenced by the optimal dietary protein-to-energy balance, the amino acid composition and digestibility of the test protein(s), and the amount of non-protein energy sources in the test diet"*. This way of perceiving protein requirements has given rise to two of the most commonly used diet optimisation tools in aquaculture, namely the 'DP:DE optimisation' ^(15, 16, 45-47) and the 'ideal protein concept' ⁽⁴⁸⁻⁵⁰⁾. Here the optimal DP:DE ratio is regarded as the minimum amount of DP required for optimizing a certain production trait such as growth, feed conversion or protein retention at a given DE density. Furthermore, the ideal protein concept is a way of tailoring a dietary amino acid profile to fit the requirements of a specific species at a specific life-stage.

At a first glance, fish generally appear to have much higher protein requirements than other vertebrates, as reflected in diets for aquacultured carnivorous species containing between 40 to 50% protein. This is roughly double of the content in diets for other vertebrates⁽⁴⁷⁾. However, when expressed relative to live weight gain and feed intake, only small differences in protein utilisation are apparent⁽¹⁵⁾. In contrast, the feed conversion efficiency of fish is roughly three times as efficient as in other vertebrates⁽⁴⁷⁾. This can be explained by the fact that fish compared to most other animals save substantial amounts of metabolic energy through mainly three strategies: 1) Avoiding spending energy on increasing body temperature above ambient temperature (being poikilothermic); 2) Being efficiently supported by surrounding water fish saves energy moving and keeping body posture; and 3) Not converting toxic nitrogenous waste from protein catabolism to urea or uric acid (as mammals and birds do), but excreting this passively over the gills at little or no energetic cost. Combined, these energy saving measures allow for fish to allocate a much larger fraction of the available metabolic energy for growth. Thus, what appears to be a high protein requirement is actually more related to a very energy efficient deposition of protein, which

consequently increases the optimal dietary protein-to-energy ratio for fish compared to other farmed animals.

Gilthead sea bream has been perceived to have a high dietary protein requirement^(51, 52), and relatively poor protein utilization⁽¹³⁾ and feed conversion compared to other aquacultured species such as salmonids. This is also reflected in the reported optimal DP:DE ratios for this species^(53, 54), which are considerably higher than for farmed salmonids at a comparable life-stage⁽⁵⁵⁻⁵⁷⁾. Irrespective of species, practically all DP:DE studies reported so far have focused on optimizing protein retention. In practice, this is typically done by reducing the dietary DP:DE level by substituting DE supplied from DP with DE supplied from non-protein DE sources such as fat⁽⁵⁸⁻⁶¹⁾ or carbohydrates^(59, 62-64).

Recommended dietary DP:DE ratios and essential amino acid requirements are depicted in figure 6 and 7.

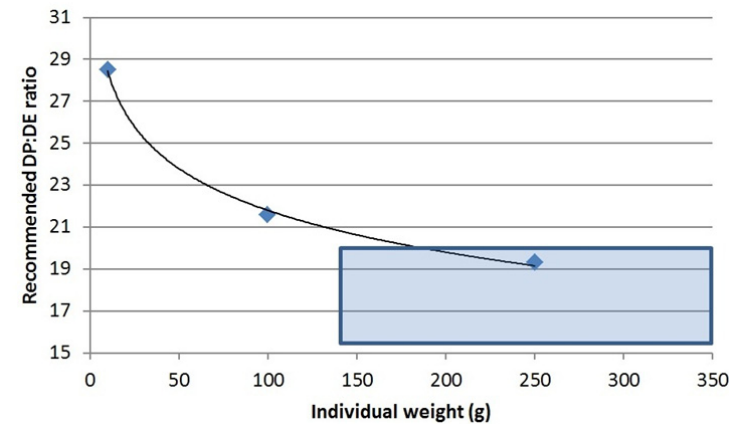


Figure 6. Dietary DP:DE levels recommended by Lupatsch *et al.* (2001) for growing gilthead sea bream. The blue box indicates the DP:DE span tested in paper 2.

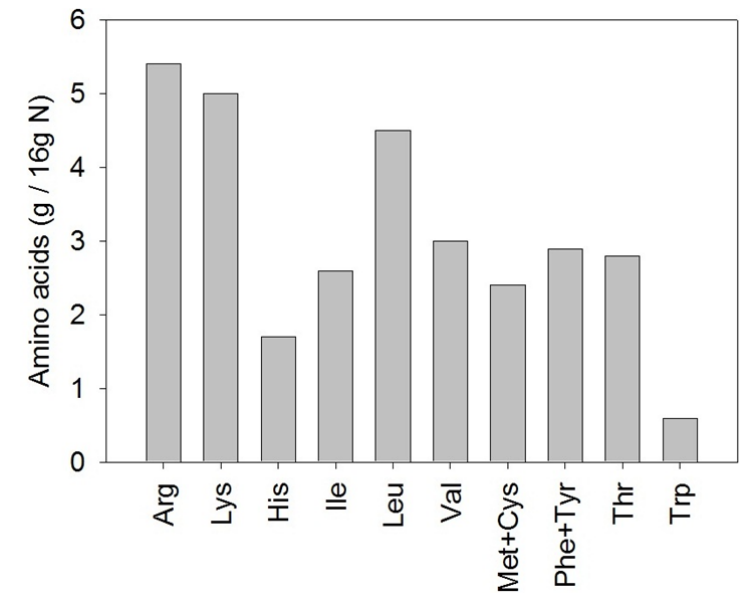


Figure 7. Essential amino acid requirements expressed as a fraction of total nitrogen (protein) as recommended by Kaushik (1998).

As apparent from figure 6 the DP:DE ratios tested in paper 2 are in the low end of what was recommended by Lupatsch *et al.* (2001). The results of paper 2 suggest that gilthead sea bream may be capable of utilising diets with lower DP:DE ratios than previously recommended without adverse effects on performance or body composition. Furthermore the results showed that the DP retention efficiency was improved with decreasing DP:DE ratio. These results corroborate the direct relationship between dietary DE level and FCR seen in this^(53, 54) and other species^(58, 65).

1.3.3 Carbohydrates

Fish have a mandatory demand for carbohydrates (glucose) as a source of energy for certain organs and tissues, such as the central nervous system, blood cells and gonad tissue. However, in aquacultured species this is not reflected in a dietary demand for carbohydrates, because fish are capable of synthesising glucose from other metabolites endogenously (gluconeogenesis)⁽⁶⁶⁾. The natural diet of most aquacultured carnivorous species is more or less devoid of carbohydrates, comprising basically only proteins and lipids as energy yielding nutrients. In contrast, commercial aquaculture diets typically comprise considerable amounts of carbohydrates, mainly because of their excellent binding capacity (important in the feed extrusion process), and because they are by far the cheapest source of dietary energy. Carbohydrates can be divided in to two groups: 1) simple carbohydrates, and 2) complex carbohydrates. The simple carbohydrates comprise mono-, di- and oligo-saccharides and sugar alcohols (e.g. glycerol), while the complex carbohydrates comprise starch, glycogen, fibres and carbohydrates in complex with proteins (glycoproteins), amines (glucosamines) or lipids (glycolipids). In aquaculture diets starch is generally considered to be the most important carbohydrate source.

Upon ingestion, dietary starch is digested in the anterior part of the intestine, where the pancreatic tissues excrete α -amylase and α -glucosidase (carbohydrases) which through a series of processes degrade starch into di- and mono-saccharides. Di-saccharides are subsequently cleaved to mono-saccharides by brush border enzymes in the mucosal membrane, after which mono-saccharides are transported to the mucosal cells either actively or passively. Enzymes involved in the degradation of starch have been found in a number of cultured species ⁽⁶⁷⁻⁷⁰⁾, including gilthead sea bream⁽⁶⁷⁾. In addition, chitinase and cellulase have been isolated from the digestive system of certain fish species, but it is unclear if these are excreted endogenously or originate from microorganisms within the fish intestine⁽⁷¹⁾. Generally, intestinal amylase activity depends on the natural diets of a fish species, thus

herbivorous and omnivorous species show higher amylase activity than carnivorous species⁽⁷⁰⁾, and are consequently able to digest higher dietary concentrations of starch. The efficiency with which starch is digested from a diet depends on a number of factors such as: fish species⁽⁶⁸⁾, water temperature^(68, 71), dietary inclusion level^(13, 64, 72) and complexity, nature, refinement and processing of the starch^(12, 62, 73, 74). After entering the blood stream, sugars (primarily glucose) are transported *via* the portal vein to the liver where they are either utilised as energy, stored as glycogen or converted to lipid or other metabolites such as glucogenic amino acids and pentoses. Glucose can be utilised for energy either aerobically or anaerobically. Common for both pathways is the glycolysis (figure 8) through which glucose *via* a number of processes is converted to pyruvate (in the ratio 1:2). Pyruvate is subsequently converted into either lactate (anaerobic) yielding a net energy of two adenosine triphosphate (ATP), or acetyl-CoA (aerobic) which through the TCA cycle and the electron transport chain undergoes complete combustion yielding a net energy of 36 ATP. If not utilised for energy purposes directly, the yielded acetyl-CoA may alternatively be converted to non-essential AAs or participate in *de novo* lipogenesis (pathways described in section 1.3.2 and 1.4).

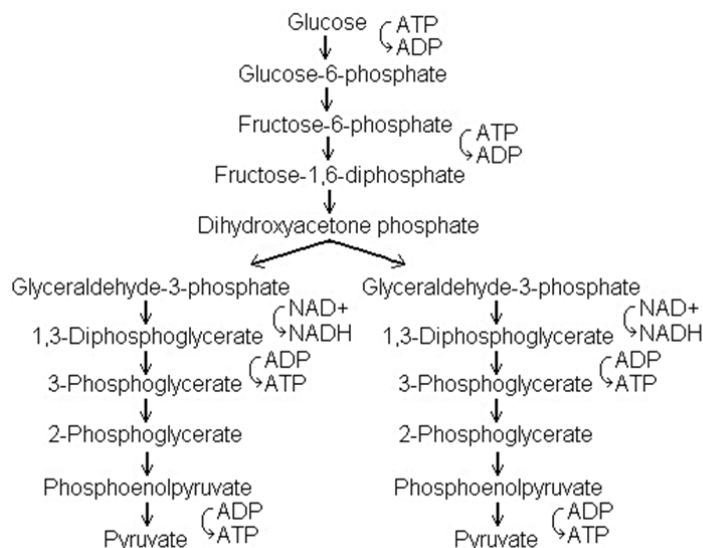


Figure 8. The steps of glycolysis converting carbo-hydrates into pyruvate. Notice that the process can be reversed yielding glucose from pyruvate (*via* acetyl-CoA and oxalo-acetate) in a process termed gluconeogenesis (please also refer to figure 9). Modified from Stryer (1988)⁽⁷⁵⁾.

Fish in general, and carnivorous fish in particular, are considered to have a limited capacity to utilise dietary carbohydrates^(11, 12, 14). In spite of this, several studies on gilthead sea bream have reported excellent starch digestibility coefficients (in excess of 95%)^(18, 64, 76, 77) as well as presence and activity of the major enzymes of the glycolytic pathway⁽¹⁸⁻²¹⁾, indicating a

good uptake and the potential for efficient utilization of dietary starch for energy purposes. However, a prolonged, postprandial hyperglycemia and accumulation of glycogen in muscle and liver tissues^(18, 19, 21, 78) suggests an imbalance between digestive/glycogenetic and glycogenolytic/glycolytic activity associated with feeding high carbohydrate diets to this species. The results from paper 1 of this thesis suggest that this imbalance could be attributed to a lack of dietary regulation of FbPase in the glycolytic pathway (figure 8 and 9), corroborating the findings of several other researchers^(18-20, 22, 25). The continuous supply of glucose from both exogenous (dietary) and endogenous (gluconeogenesis) sources appear to cause excessive glycogenesis, resulting in an accumulation of glycogen in both whole fish and livers, which in turn have led to hepatomegaly in fish fed high carbohydrate diets^(11, 13, 79).

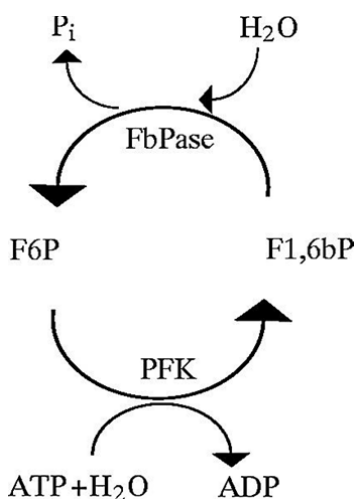


Figure 9. The major control site for regulation of glycolysis and gluconeogenesis. The activity of the enzymes fructose 1,6-bisphosphatase (FbPase) and phosphofructokinase (PFK) promote gluconeogenesis and glycolysis, respectively. Both enzymes are allosterically affected by fructose 2,6-bisphosphatase, which consequently plays an important regulatory role in carbohydrate degradation or formation through the glycolytic pathway⁽²²⁾.

Generally, a maximum digestible carbohydrate inclusion level of 20% has been recommended for marine fish and salmonids, while up to 40% may be included in diets for warm water omnivorous species^(12, 14). Enes *et al.* (2011) recommended a maximum inclusion level of 20% digestible carbohydrate in diets for juvenile gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*).

1.3.4 Lipids

Lipids are a diverse group of molecules which in both plants and animals serve as an important source of metabolic energy, particularly for storage purposes. While carbohydrate and protein hold 17 and 24 MJ energy/kg, respectively (upon complete oxidation) lipid holds 39 MJ energy/kg (upon complete oxidation)⁽⁸⁰⁾. In addition, lipids have a low mass density (approximately 900 g/l) compared to protein (approximately 1060 g/l), due to the fact that

lipid, in contrast to proteins and carbohydrates, does not bind water upon storage. Combined, this makes lipid the ideal energy storage nutrient. In addition, lipids are important carriers for lipid soluble nutrients such as certain vitamins and carotenoids. Depending on the structural or functional properties lipid can be classified into different groups: triacylglycerols (triglycerides) with three individual fatty acids esterified onto a glycerol molecule, glycerophospholipids (phosphoglycerides) with two individual fatty acids esterified onto a glycerol molecule. In contrast to triacylglycerols, the third carbon atom of the glycerol molecule in glycerophospholipids is bound to a phosphate group, which in turn is esterified to an alcohol (e.g. choline, inositol etc.). Other lipid groups include wax esters, cholesterol, cholesterol esters and sphingolipids. Fatty acids themselves comprise a carbon chain, which in one end is carboxylised (COOH) and in the other end have a methyl group (CH₃). Fatty acids are named from the carbon chain length, the level of saturation (number of double bonds in the chain) and location of first double bond. Thus, in 20:5n-3 (eicosapentaenoic acid; EPA) '20' is the number of carbon atoms in the chain, '5' is the number of double bonds in the molecule, and 'n-3' tells that the first double bond (from the methylated end of the molecule) is located on carbon atom number 3. Naturally occurring fatty acids almost exclusively comprise an even number of carbon atoms in their chains.

As source of metabolic energy and storage compound triglycerides are the most important lipid group, while the glycerophospholipids are essential for cellular membrane structures. In fish, storage lipids are mainly found in the liver, viscera or in muscle tissue. Fish can obtain lipids from either exogenous (feed) or endogenous sources (*via de novo* lipogenesis). The latter is presented in further detail in section 1.4. In fish, lipids obtained from exogenous sources are not degraded before they have passed the stomach (if present) and reach the pyloric caeca (or anterior part of the intestine if caeca are not present). Here the drop in pH and increment of free fatty acids triggers the secretion of the hormone cholecystokinin, which in turn enhances the release of pancreatic digestive enzymes and bile acids, facilitating the formation of micelles. The micelles are microscopic lipid droplets which allows for the lipid to be blended homogeneously into the chyme. Additionally, micelles dramatically increase the surface area of ingested lipid, which in turn allows for a much larger 'attack area' for lipases to work on subsequently. Lipases further degrade these micellar lipids by breaking ester bonds of triglycerides, wax esters, phospholipids and cholesterol esters, leaving micellar contents to mainly comprise free fatty acids, diacylglycerols, monoacylglycerols, phospholipids and cholesterol. These are in turn liberated from the micelles upon contact with the 'unstirred water layer' of the intestinal epithelium (due to low pH) and diffuse passively

to the intestinal mucosa and into the enterocytes. It is assumed that fish digests lipids over the full length of the intestine. However, the majority of lipid is believed to be digested from the anterior part of the intestine to the mid intestine⁽⁸¹⁾. The overall lipid digestibility in fish is generally high (around 90%), but digestibility is highly affected by fatty acid saturation and chain length. Thus, while polyunsaturated fatty acids (PUFAs) display apparent digestibility coefficients (ADC) around 95%, ADCs of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SAFAs) are much lower (approximately 85 and 70%, respectively). Lipid ADCs are similarly reduced with increasing fatty acid chain length⁽⁸¹⁾. In the intestinal mucosal cells fatty acids are re-esterified mainly into triglycerides and phospholipids upon digestion.

In the blood, lipids are mainly transported in the form of lipoproteins grouped according to their protein-to-lipid ratios and size: chylomicrons, very low density lipoproteins (VLDL; high lipid-to-protein ratio), low density lipoprotein (LDL), high density lipoprotein (HDL) and very high density lipoproteins (VHDL; low lipid-to-protein ratio)⁽⁴⁰⁾. These are then transported to peripheral tissues or liver for deposition, energy production or structural purposes.

Oxidation of lipids for energy purposes takes place mainly in the liver and in the red muscle tissue of fish⁽⁸¹⁾. Before triglycerides can be oxidised for energy, they have to be cleaved into glycerol and individual fatty acids. Grossly simplified, glycerol is subsequently converted into dihydroxyacetone phosphate, which in turn can enter the glycolytic pathway. The residual fatty acids are oxidised in the mitochondria in a process called β -oxidation, which cleaves acetyl-CoA units sequentially from the fatty acid chain, and in addition yields both NADH and FADH₂. Acetyl-CoA could then be oxidised further through the TCA cycle for energy, or be utilised for other metabolic purposes as described in section 1.3.2. The combined energy yield from acetyl-CoA, NADH and FADH₂ when breaking down a 18:0 fatty acid is equivalent to 148 ATPs⁽⁸¹⁾.

Certain long-chained poly unsaturated fatty acids (PUFAs) are mandatory biological participants in a number of metabolic and physiological processes, such as eicosanoid production, vision, brain development and regulation of expression of genes involved in lipid metabolism⁽⁸²⁾. These include 20:4n-6 (Arachidonic acid; ARA), 20:5n-3 (Eicosapentaenoic acid; EPA) and 22:6n-3 (Docosahexaenoic acid; DHA), which are marked in bold in figure 10. As $\Delta 12$ and $\Delta 15$ fatty acyl desaturases (marked in red in figure 10) are active only in plants and certain invertebrates, 18C FAs of the n-3 and n-6 series and elongated homologues cannot be biosynthesised *de novo* from 18C saturated fatty acids (SAFAs) or mono-

unsaturated fatty acids (MUFAs). Also, vertebrates are unable to interconvert the n-3 and n-6 PUFA series⁽⁸³⁾. Thus, lipids synthesised *de novo* from substrates other than lipid (such as protein or carbohydrates) cannot be converted into PUFAs. As a consequence, these have to be supplied through the diet, and they are therefore termed essential fatty acids (EFA). The extent to which different fish species are able of elongating and desaturating 18C PUFAs to ARA, EPA and DHA differs. In freshwater and diadromous fish species the EFA requirement can apparently be satisfied from elongation and desaturation of the 18C PUFAs of the n-3 and n-6 series (linolenic and linoleic acids, respectively - marked in italics in figure 10), while marine fish do not appear to be able to satisfy the EFA requirements by converting 18C PUFAs⁽⁸³⁾. Thus, in culture of marine fish ARA, EPA and DHA need to be supplied directly into the diet to assure fulfilment of EFA requirements. The EFA requirements of juvenile (not larvae/fry) gilthead sea bream is reported between 0.9 to 1.9% of the dry diet, with a DHA-to-EPA ratio between 0.5 and 1.0⁽⁸⁴⁻⁸⁶⁾.

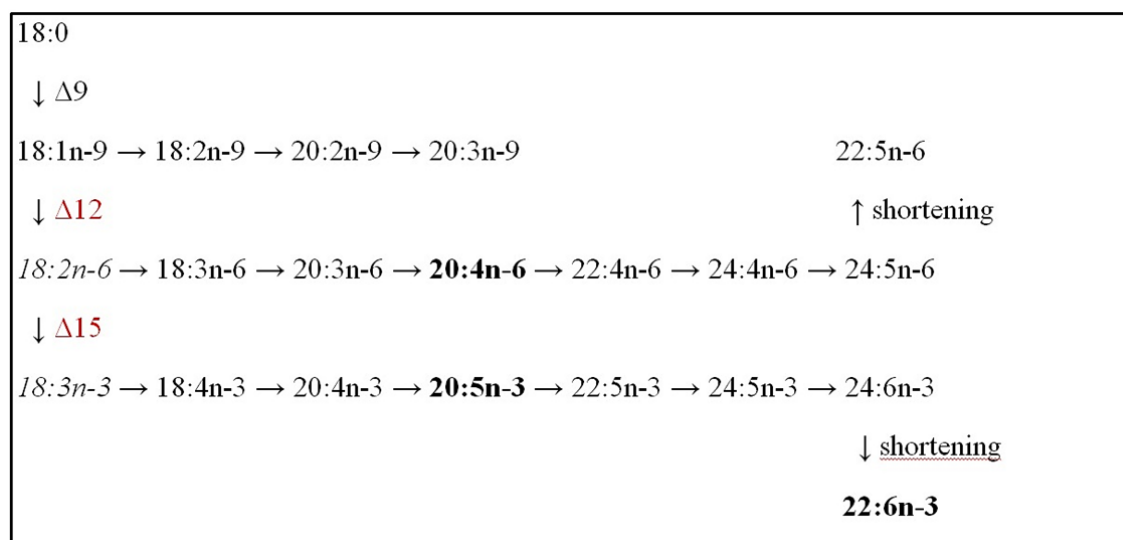
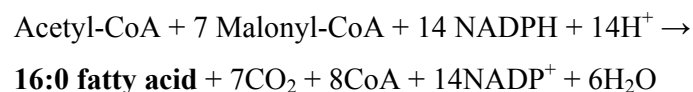


Figure 10. Pathways of biosynthesis of long chained PUFAs from n-3, n-6 and n-9 18C PUFA in fish. Modified from Tocher (2003)⁽⁸³⁾.

1.4 *De novo* lipogenesis

Just like other vertebrates fish are not able to store excess amino acids or carbohydrates to any significant extent, which seen from an energy conserving point of view may appear uneconomical. However, as already introduced in the preceding sections all of the major nutrients have the ability to precede one common metabolite, namely acetyl-CoA, which in

combination with the reducing powers of NADPH enables the process of *de novo* lipogenesis. Thus, *de novo* lipogenesis is the process by which acetyl-CoA is endogenously converted into lipids, and serves as a clever way of conserving valuable dietary energy reserves from different groups of macronutrients when these are not immediately needed for other structural or energetic purposes. The process is reversible, allowing regeneration of metabolites (such as acetyl-CoA, NADH and FADH₂) from deposited lipid *via* β -oxidation. The process of lipogenesis in fish is believed to be very similar to that of mammals. However, in contrast to mammals lipogenic activity appears to mainly occur in the liver, whereas lipogenesis in mammals primarily takes place in the adipose tissues.^(87, 88) The chemical pathway of lipogenesis is catalysed by fatty acid synthetase (FAS) multienzymes complex⁽⁸⁹⁾, and the main products of the process are 16:0 (palmitic acid) and 18:0 (stearic acid) fatty acids. The process requires an acetyl-CoA unit serving as a primer, and sequential addition of malonyl-CoA units (obtained from carboxylation of acetyl-CoA) combined by the FAS complex in a process requiring the reducing powers of NADPH. The overall chemical reaction for formation of fatty acids *de novo* is⁽⁸⁸⁾:



1.4.1 Sources of acetyl-CoA

The acetyl-CoA participating in *de novo* lipogenesis can arise from a number of biochemical processes which degrade carbohydrates, protein or lipids yielding Acetyl-CoA as an end product (also see figure 5):

1.4.1.1 Acetyl-CoA originating from amino acids

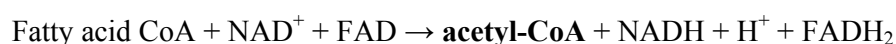
Of the 20 amino acids derived from protein five potentially yield acetyl-CoA directly: phenylalanine, tyrosine, leucine, lysine and tryptophan, and other five can yield acetyl-CoA via pyruvate: alanine, threonine, glycine, serine and cysteine (figure 5). The ten amino acids broken down to acetoacetyl-CoA / acetyl CoA can yield either ketone bodies or donate carbon for lipogenesis (ketogenic amino acids), while the amino acids entering the TCA cycle *via* α -ketoglutarate, succinyl-CoA, fumarate or oxaloacetate are potential carbon donors in gluconeogenesis (glucogenic amino acids). Besides participating in gluconeogenesis, lipogenesis and ketogenesis all amino acids can be completely oxidized for energy purposes, or as mentioned previously, participate in protein synthesis.

1.4.1.2 Acetyl-CoA originating from carbohydrates

Also carbohydrates can yield acetyl-CoA. This is accomplished through glycolysis, which is principally a process where glucose *via* a series of biochemical steps is converted into pyruvate, in the ratio 1:2, respectively (figure 8). Pyruvate is then converted to acetyl-CoA, which in turn can be either fully oxidised *via* the TCA cycle and electron transport chain or participate in lipogenic or ketogenic pathways. Pyruvate is an important metabolic junction, because pyruvate is capable of preceding both gluconeogenesis (re-generate glucose) and acetyl-CoA. However, upon conversion to acetyl-CoA, there is no returning to pyruvate/carbohydrate, and thereby no way back into the gluconeogenetic pathway.

1.4.1.3 Acetyl-CoA originating from lipids

The principles of the degradation of fatty acids and their residual products were described in section 1.3.4 (β -oxidation). The overall reaction is:



1.4.2 Sources of NADPH

The reducing powers (in the form of NADPH) required in the process of lipogenesis can be obtained from three different processes in vertebrates.

- 1) NADPH can be generated in the oxidative phase of the pentose phosphate pathway, in which glucose-6-phosphate dehydrogenase catalyses the oxidation of glucose-6-phosphate and NADP^+ serves as the electron donor:



- 2) NADPH can be generated by means of malate dehydrogenase which catalyses the oxidation of malate to pyruvate, with which NADPH and CO_2 are produced concomitantly:



- 3) NADPH can be generated by the oxidative decarboxylation of isocitrate (catalysed by isocitrate dehydrogenase), producing α -ketoglutarate, NADPH and CO_2 :



The activity of the three NADPH generating pathways differs greatly between species. Thus, while NADPH appears to be mainly provided by the oxidation of malate to pyruvate in salmon⁽⁹⁰⁾, NADPH is mainly supplied by the oxidation of glucose-6-phosphate (pentose

phosphate pathway) in gilthead sea bream⁽⁹¹⁾, European seabass (*Dicentrarchus labrax*)⁽⁶³⁾ and rainbow trout (*Onchorynchus mykiss*)^(92, 93).

1.5 Stable isotopes as metabolic tracers in nutritional studies

1.5.1 History

The term isotope was first suggested by the Scottish physician Margaret Todd to Frederick Soddy, a chemist at Glasgow University in 1913. Dr. Soddy had from some of his research discovered that it seemed as if multiple species of certain atoms existed, with the same number of protons, but different atomic weights. Therefore, Dr. Todd suggested the Greek term for ‘at the same place’ (=isotope) to describe these discoveries. The first one to actually report the existence of naturally occurring isotopes, however, was J.J. Thomson who discovered two stable isotopes of neon.

1.5.2 Theory

A basic element contains a fixed number of protons in its core, while the number of neutrons can differ. Taking the carbon (C) atom as an example, the most abundant isotope of this basic element is $^{12}_6\text{C}$, meaning that the atom consists of 6 protons, 6 neutrons (i.e. atomic weight 12u) and 6 electrons. The atomic weight is 12u by definition. The mass of both protons and neutrons changes depending on whether the particles are ‘free’ or if they are ‘bound’ in an atom. It is therefore not possible to *calculate* the exact mass of an atom, but it is possible to *measure* it on a mass spectrometer. Isotopes of basic elements can be either stable or unstable (radioactive). It is the number of neutrons in the nucleus of an atom that determines if it is stable or not. Protons in the nucleus are positively charged, meaning they will repel each other. The presence of (non-charged) neutrons separates the protons slightly, making the core stable. In the case of carbon there are two stable isotopes: $^{12}_6\text{C}$ and $^{13}_6\text{C}$, and one radioactive isotope $^{14}_6\text{C}$. Here, the surplus of neutrons makes the $^{14}_6\text{C}$ unstable (6 protons and 8 neutrons). This causes the atom to spontaneous decay, and one of the neutrons becomes a proton under the release of an electron: $^{14}_6\text{C} \rightarrow ^{14}_7\text{N} + ^0_{-1}\text{e}$

The resulting electron is emitted as a beta ray at the speed of light. The half-life of $^{14}_6\text{C}$ is approximately 5730 years.

In contrast to radioactive isotopes, there is no spontaneous decay of stable isotopes. Stable isotopes of an atom contain variable numbers of neutrons in the nucleus (see table 1).

Table 1. Examples of stable isotopes

Element	No. of protons+neutrons	% Natural abundance
Hydrogen (H)	1	99,985%
	2	0,015%
Carbon (C)	12	98,89%
	13	1,11%
Nitrogen (N)	14	99,63%
	15	0,37%
Oxygen (O)	16	99,76%
	17	0,037%
	18	0,204%
Sulphur (S)	32	95,00%
	33	0,76%
	34	4,22%

1.5.3 Stable isotopes as tracers

Stable isotopes have been used as metabolic tracers approximately 20 years before radioactive isotopes. The first studies were conducted at Columbia University in 1935 where Schoenheimer and Rittenberg⁽²⁸⁾ used the stable isotope of hydrogen ²H (deuterium) to study fat metabolism in mice. Other research fields where isotope ratio mass spectrometry (IRMS) analysis is applicable include geochemistry, hydrology, atmospheric chemistry and glaciology.

The reason that isotopic variations occur in nature, is because substances or chemical or biological processes preferentially concentrates one isotope over another. The difference in isotopic composition between a sample and a reference is determined by the equation:

$$[(R_{\text{sample}} - R_{\text{standard}}) / (R_{\text{standard}})] \times 1000 = \delta_{\text{sample-standard}}, \text{ where}$$

R_{sample} is the ratio of the heavy isotope to light isotope in the sample, R_{standard} is the ratio of the heavy isotope to light isotope of the reference (which is calibrated against an internationally known standard (see table 2)), and $\delta_{\text{sample-standard}}$ is the difference in isotopic composition of the sample relative to the reference expressed in ‰.

In biological studies stable isotopes can be used as tracers in order to determine complex biochemical pathways. The perfect tracer is a compound which behaves functionally identical (not metabolically discriminated) to the tracee (the compound of interest), but still can be distinguished from the tracee by measurement. Radioactive isotopes can be distinguished

from the more abundant form on an element by radiometric measurements. Stable isotopes can be distinguished by mass spectrometry.

In metabolic studies the most commonly used tracers are the heavy forms of the atoms H, C, N, O and S, since these elements basically make up all organic matter. Supersaturated (with the heavier isotope) versions of these elements can be incorporated into molecules of interest (e.g. amino acids, fatty acids or carbohydrates). These molecules can be either fully ‘saturated’, meaning that the tracer is replacing basically all of the tracee in the compound (e.g. a fatty acid where all carbon atoms in the molecule is replaced) or it is possible to target the tracer to a specific position in a molecule (e.g. only carbon in position 1 in a cholesterol molecule is labelled). The latter can be extremely useful when investigating complex biochemical pathways, since the fate of each atom in a molecule can be determined.

Table 2. International Rstandardvalues.

Standard	R _{standard}	Element
V-SMOW ¹⁾	0.0001557	² H
V-PDB ²⁾	0.011056	¹³ C
V-SMOW ¹⁾	0.0020004	¹⁸ O
N AIR ⁴⁾	0.003663	¹⁵ N

¹⁾V-SMOW (standard mean ocean water) – used for $\delta^2\text{H}$ and $\delta^{18}\text{O}$ isotope measurements. ²⁾V-PDB (Pee Dee Belemnite) – used for $\delta^{13}\text{C}$ measurements. N AIR (Atmospheric Nitrogen) – used for $\delta^{15}\text{N}$ measurements.

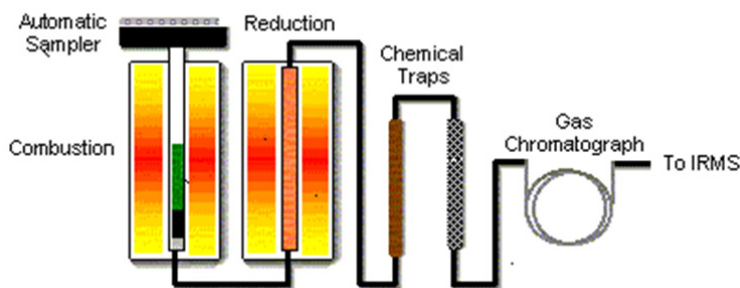
1.5.4 Principles of IRMS analysis

1.5.4.1 The Elemental analyser (EA)

Even though the principles of these analyses are quite simple, the procedures needed to do so are rather complex; the samples are dried and packed into tin capsules and weighed on a mg scale with 3 decimals. Samples are loaded into the auto sampler carousel where they will be automatically dropped into the elemental analyzer. Information about sample weights and placement in the auto sampler is entered into the Isodat NT 3.0 software. In the elemental analyzer (Figure 11) samples are dropped into a 1050°C combustion column where the tin capsule packing will cause a ‘flash combustion’ where the sample will reach 1700°C instantaneously, causing the sample to become an oxidized gaseous product and ash. The gaseous product will throughout the whole analysis process be carried by an inert gas (He) through the system(s). The lower half of the combustion column is packed with chromium oxide and cobaltous oxide (cobalt(II)oxide) which cause a full oxidation of the gaseous product, forcing all carbon to be on the CO₂ form. During the whole oxidation step pure

oxygen is introduced into the column, assuring full oxidation as well as partial regeneration of the chromic oxide in the combustion column. Next step in the process is the copper column (column packed with copper granules and copper oxide) where nitrous oxides (NO_x) are reduced, causing all nitrogen to be converted to the N_2 form and assure removal of excess O_2 . Next step is a water trap where all water is removed from the gaseous sample. The water trap can, if necessary, be coupled in serial to a CO_2 trap (Carbosorb) if carbon analysis is not needed from the sample. At this point the sample is a dried gaseous mix of N_2 , CO_2 and carrier gas. Next step in the process is separation of N_2 and CO_2 in a GC column. Due to differences in molecular size and polarity of CO_2 and N_2 , the GC column will release N_2 faster than CO_2 . CO_2 will, in other words, have a longer retention time in the GC column. After GC separation the gasses pass a thermal conductivity detector (TCD) which *can* be used for quantification of CO_2 or N_2 .

Figure 11. Illustration of the principles of the elemental analyzer



1.5.4.2 The ConFlo IV interface between the EA and the IRMS

The ConFlo IV (Figure 12) is an advanced gas mixing device which continuously feeds a gas mix into the IRMS. Gasses are mixed in a so-called open split setup which basically functions as an open 'room' (cell) in which gasses are continuously mixed and where a diverting capillary leads the mixed gasses to the IRMS for analysis. Besides the sample gas mix (sample gas and carrier gas) the open split allows further addition of carrier gas (He) for dilution of sample gas mix. Also, it is in the open split reference gasses are added (in this case reference CO_2 and N_2 – not shown in figure 12). Which gasses are mixed at what time require careful timing both concerning arrival of sample gas from the EA and exit of mixed gas to the IRMS. It is this timing that makes it possible to analyze multiple elements (N and C) and their isotopic composition from combustion of a single sample.

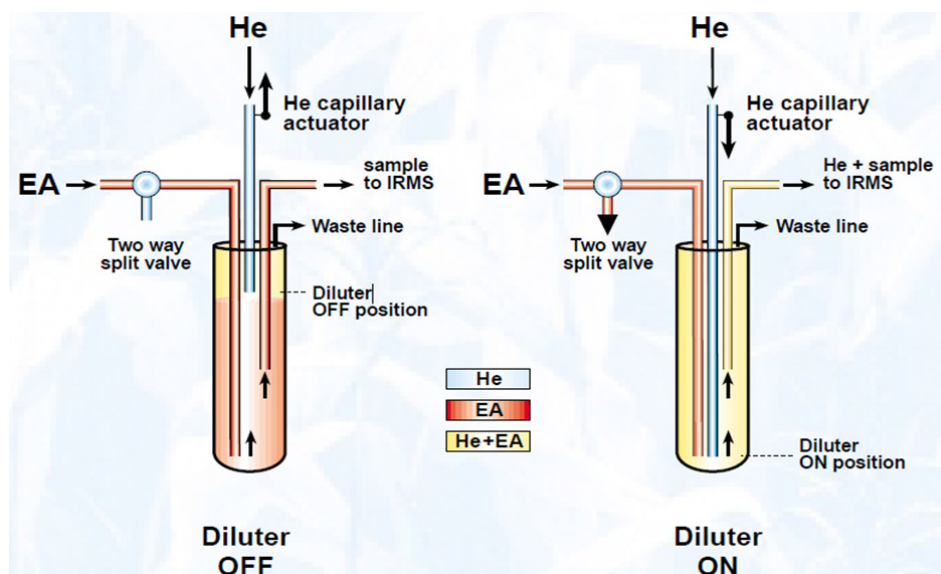


Figure 12. Illustration of the principles of the open split configuration (ConFlo IV interface)

1.5.4.3 The Isotope Ratio Mass Spectrometer (IRMS):

The IRMS is where the quantification and isotopic abundance of N and C takes place by separating charged molecules by mass. The IRMS comprise three basic sections: an ion source, a mass analyzer and an ion collection setup. The gaseous sample is introduced into an ionization chamber where interaction with a focused electron beam causes electrons to be removed from the molecules, resulting in the formation of positive ions (eg. CO_2^+). The ions are accelerated (by a voltage difference of $\sim 3000\text{V}$ between ion source and collector cups) out of the chamber, through a flight tube placed between the poles of a strong electro magnet. Here the ionized molecules are separated according to their mass to charge ratio (m/z) and collected by a row of three Faraday cup collectors (see figure 13). All processes running in the IRMS takes place under extreme vacuum conditions (10^{-6} bar). When hit by an ionized particle the collector is electrically charged. The magnitude of this electrical signal is proportional to the number of ions striking the detector. It should be noticed that when measuring $^{13}\text{C}/^{12}\text{C}$ ratios certain algorithms are needed in the computer software used for interpretation of results. This is because a similar weight of the measured CO_2^+ ions can be obtained by several different combinations of ‘heavy’ and ‘light’ oxygen and carbon atoms. Please refer to examples in table 3.

Table 3. Different isotopic combinations of CO_2^+ .

Possible atomic composition of particle CO_2^+	Weight (u)
$^{12}\text{C}^{16}\text{O}^{18}\text{O}$	46
$^{12}\text{C}^{17}\text{O}^{17}\text{O}$	46
$^{13}\text{C}^{16}\text{O}^{17}\text{O}$	46
$^{12}\text{C}^{16}\text{O}^{17}\text{O}$	45
$^{13}\text{C}^{16}\text{O}^{16}\text{O}$	45
$^{12}\text{C}^{16}\text{O}^{16}\text{O}$	44

Even though the latter two combinations in the table are the most abundant ones by far, the computer software ‘corrects’ the obtained results, so effects of the other combinations are minimized when determining $^{13}\text{C}/^{12}\text{C}$ ratios.

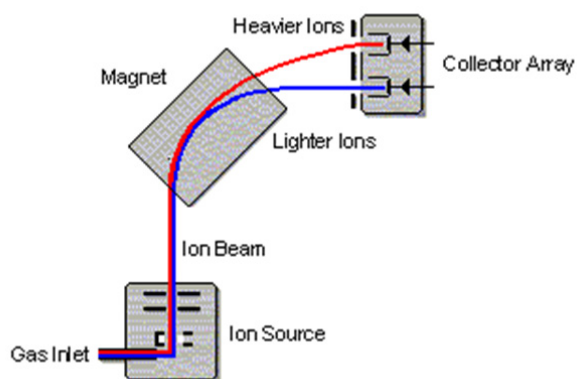


Figure 13. Illustration of the principles of the IRMS

2. Paper I: Glycogenesis and *de novo* lipid synthesis from dietary starch in juvenile gilthead sea bream (*Sparus aurata*) quantified with stable isotopes

**Glycogenesis and *de novo* lipid synthesis from dietary starch in juvenile gilthead sea bream
(*Sparus aurata*) quantified with stable isotopes**

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Running title: Starch utilization in gilthead sea bream

Keywords: gilthead sea bream: stable isotopes: starch utilization: glycogenesis: lipogenesis

1. Abstract

The effects of replacing digestible energy (DE) source from fat (fish oil) to carbohydrate (wheat starch) on performance, glycogenesis and *de novo* lipogenesis was examined in triplicate groups of juvenile gilthead sea bream, fed four extruded experimental diets. In order to trace the metabolic fate of dietary starch, 0.7% wheat starch was replaced with isotope labelled starch (>98% ^{13}C). Fish were fed the experimental diets for 3 consecutive 10 day periods, and isotope ratio mass spectrometry was applied to quantify ^{13}C enrichment of liver and whole body glycogen and lipid pools over the 3 feeding periods. Glycogenesis originating from dietary starch accounted for up to 68.8 and 38.8% of the liver and whole body glycogen pools, respectively, while up to 16.7% of the liver lipid could be attributed to dietary starch. Between 5 – 8% of dietary starch carbon was recovered in whole body lipid, and estimated deposition rates of *de novo* synthesized lipid originating from starch ranged from 18.7 to 123.7 mg/kg biomass/day. Dietary treatments did not significantly affect growth, feed performance or body composition of the fish, while the hepatosomatic index and glycogen content of whole fish and livers correlated directly with dietary starch inclusion level. The study suggests that gilthead sea bream efficiently synthesizes glycogen from both dietary starch and endogenous sources. In contrast, lipogenesis from carbon derived from starch seems to play a minor role in the overall lipid synthesis and deposition under the specified experimental conditions.

2. Introduction

The rapid growth in the aquaculture industry over the last thirty years and the concomitant increasing demand for compound aquaculture feeds has adversely influenced both commodity prices and raised sustainability issues concerning utilization of natural resources from industrial fisheries such as fish meal and fish oil^(1, 2). As a consequence, a substantial number of studies have been carried out to elucidate the nutritional value of alternative nutrient sources such as vegetable proteins⁽³⁾ and oils^(4, 5), animal by-products^(6, 7), or single cell organisms⁽⁸⁻¹⁰⁾ as replacements for fish meal or fish oil in commercial aquaculture diets. Although many of these alternatives have proven to be competitive substitutes to fish meal or fish oil in terms of price cost, some may raise nutritional, safety, or ethical concerns^(3, 11-13) regarding their use in the fish feed industry.

The natural diet of most aquacultured carnivorous fish species typically has a protein and lipid rich nutritional profile, abundant in essential amino acids and fatty acids, and practically devoid of carbohydrates or anti-nutrients. Thus, introduction of dietary vegetable raw materials containing relatively high amounts of carbohydrates and possibly associated anti-nutrients, may pose a challenge to the carnivorous nature of the digestive processes in these species^(14, 15). In contrast, several studies on gilthead sea bream have reported excellent starch digestibility coefficients⁽¹⁶⁻¹⁸⁾ as

69 well as presence and activity of the major enzymes in the glycolytic pathway^(17, 19-21), indicating a
 70 possible efficient utilization of dietary starch for energy purposes. However, an apparently
 71 prolonged, postprandial hyperglycemia and accumulation of glycogen in muscle and liver tissues<sup>(17,
 72 19, 21, 22)</sup> suggests an imbalance between digestive/glycogenetic and glycogenolytic/glycolytic activity
 73 associated with feeding high carbohydrate diets to this species. Studies on dietary carbohydrate
 74 utilization in gilthead sea bream have focused on effects of starch origin, processing and inclusion
 75 levels on carbohydrate digestibility, growth performance, feed conversion or nutrient retention
 76 efficiencies^(16, 18, 23), while other studies have also assessed the regulatory effects of dietary digestible
 77 carbohydrate on activity of liver enzymes involved in glycolysis, gluconeogenesis, glycogenesis,
 78 lipogenesis and amino acid catabolism^(17, 19-21, 24-29). However, since dietary carbohydrate, besides
 79 fuelling the glycolytic pathway, can precede *de novo* lipogenesis, glycogenesis or, as recently found
 80 in rainbow trout (*Onchorynchus mykiss*), donate carbon backbones for protein synthesis⁽³⁰⁾, it is
 81 difficult to quantitatively conclude on the fate of dietary carbohydrates. Similarly, studies on the
 82 key enzymes of carbohydrate metabolism typically examine enzyme activity as an indicator of
 83 metabolite fluxes, but does not quantify end products or determine their origin.
 84 The technique of using stable isotopes in nutritional studies dates back almost 80 years⁽³¹⁾ and
 85 makes it possible to trace the fate of various compounds in the animal body, assuming that the
 86 tracer behaves functionally similar to the tracee but still can be distinguished from the tracee by
 87 measurement. In fish, isotope labelled carbohydrates have been previously administered to Atlantic
 88 cod (*Gadus morhua*)⁽³²⁾, rainbow trout^(30, 33), Atlantic halibut (*Hippoglossus hippoglossus*)⁽³⁴⁾, and
 89 Atlantic salmon (*Salmo salar*)⁽³⁵⁾ as a single oral bolus or administered intraperitoneally, allowing
 90 researchers to determine the metabolic fate of carbohydrates and estimate synthesis rates of their
 91 derived metabolites. More recently, also deuterated water has been employed as a tracer in a study
 92 investigating glucose metabolism of European seabass (*Dicentrarchus labrax*)⁽³⁶⁾.
 93 The purpose of the present study was to quantify the magnitude of *de novo* lipogenesis and
 94 glycogenesis from dietary starch in gilthead sea bream using ¹³C labelled starch. To achieve this,
 95 gilthead sea bream were fed four diets formulated to be similar in digestible protein (DP) and DE
 96 but gradually increasing the proportion of DE supplied from fat (fish oil) with DE from starch
 97 (wheat starch) enriched with uniformly labelled ¹³C starch. The gradual ¹³C enrichment of liver and
 98 whole body glycogen and lipid pools over a period of 30 days was used to estimate starch
 99 utilization and quantify deposition rate of starch carbon for lipogenesis, and to determine the overall
 100 significance of dietary starch in liver and whole body glycogen and lipid pools including their
 101 turnover rates.

102 3. Materials and methods

103 3.1 Culture conditions and fish

104 Gilthead sea bream with an average individual mass of approximately 75 grams were stocked into a
105 recirculated aquaculture system (RAS) comprising 12 fibre glass tanks with a volume of 800 L each
106 at a stocking density of 29 fish/tank (BioMar research facility, The North Sea Research Centre,
107 Hirtshals, Denmark). The tanks were designed to quickly and efficiently remove faeces and un-
108 eaten feed pellets from tank water *via* a central bottom drain. Externally mounted swirl separators
109 made it possible to collect and quantify un-eaten feed pellets. The trial facility was supplied with
110 filtered North Sea water with a salinity of 34 g/l, and temperature was kept at 24°C throughout the
111 experiment. Water quality was monitored daily, maintaining O₂ saturation between 80-100%,
112 ammonia below 1.0 mg/l, nitrite below 1.0 mg/l, and nitrate below 100 mg/l. pH was adjusted to 7.0
113 using sodium bicarbonate when necessary. Tanks were supplied with system water at a flow rate of
114 1200 l/tank/h. A 14 h light : 10 h dark photoperiod was maintained throughout the trial.
115 In parallel to the ¹³C enrichment trial other 12 tanks of the same RAS were stocked with gilthead
116 sea bream with an individual mass of approximately 200 grams at a stocking density of 50 fish/tank.
117 The sole purpose of these fish was to determine apparent digestibility coefficients (ADCs) of major
118 dietary nutrients and stable carbon isotopes (¹³C and ¹²C) of the diets.

119 3.2 Experimental diets

120 Four diets were prepared using the Allix² feed formulation software (A-systems S.A., France)
121 (Table 1). The four diets named A, B, C and D were formulated to contain 60, 120, 180, and 240 g
122 starch/kg feed, respectively. In all diets 0.7% of the starch was replaced by uniformly ¹³C labelled
123 (>98% ¹³C) starch (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Fish oil and
124 cellulose were used to balance dietary energy levels. The diets were supplemented with a vitamin
125 and mineral premix, as well as mono calcium phosphate. Guar gum was added (5 g/kg) to enhance
126 pellet stability and accurately quantify feed waste, and yttrium oxide (Y₂O₃) was added (0.3 g/kg)
127 as an inert marker enabling indirect measurements of nutrient and stable isotope digestibility. Diets
128 were prepared at the Danish Technological Institute (Sdr. Stenderup, Denmark) using a twin screw
129 extruder (Werner & Pfleiderer Contina 37, Tamm, Germany) to produce 3 mm pellets. Following
130 extrusion, diets were dried, vacuum coated with fish oil, and cooled.

131 3.3 Experimental procedures

132 The trial comprised three consecutive feeding periods of 10 days each (30 feeding days in total),
133 feeding each of the four experimental diets to three replicate tanks (i.e. 12 tanks in all). Six
134 randomly chosen fish were removed from each of the 12 tanks at start-up (day 0), euthanized using
135 250 mg/l tricaine methanesulfonate (MS-222), weighed, and immediately frozen in liquid nitrogen.

136 The fish were pooled on tank level and stored at -20°C until analysis, constituting the initial fish
137 samples. Remaining fish in each tank were weighed, and the four experimental diets were
138 subsequently fed to triplicate tanks for 10 days at a daily ration adjusted according to the expected
139 biomass in each tank (approximately 1.3%/d). Fish were fed from 08:00 to 14:00 h using automatic
140 belt feeders, and any un-eaten feed was collected daily to calculate the actual feed intake (FI). At
141 the end of the feeding period, fish were starved for 24 h (day 11) and 6 randomly chosen fish were
142 removed from each tank (day 12), euthanized, weighed, and immediately frozen in liquid nitrogen.
143 The fish were pooled on tank level and stored at -20°C until analysis, constituting fish samples after
144 10 days of isotopic enrichment. The same procedures were followed during feeding period 2 (day
145 13-22) and 3 (day 25-34), yielding a total of 4×12 fish samples throughout the trial. The five
146 remaining fish in each tank at the end of the trial (day 34) were weighed, euthanized and discarded.
147 Apparent digestibility coefficients of major dietary nutrients and stable carbon isotopes (¹³C and
148 ¹²C) were determined using the stripping/inert marker method⁽³⁷⁾ with yttrium as the inert marker.
149 The trial was carried out on larger (200 g) fish in order to obtain sufficient quantities of faeces for
150 chemical analysis, and because larger fish are easier to handle, reducing the risk of contaminating
151 samples with mucus, urine and scales. The four experimental diets were fed to triplicate tanks for 3
152 weeks applying a feeding ration of 1.5% of the estimated biomass/d. The final meal was
153 administered 18 h prior to stripping, when the fish were anaesthetised using MS-222 (50 mg/l), and a
154 gentle bi-lateral pressure was applied to the hindgut in order to provoke defecation. Faeces obtained
155 from fish within each tank was pooled and immediately frozen at -20°C.
156 All studies were carried out in accordance to EC directive 86/609/EEC for animal experiments⁽³⁸⁾.

157 *3.4 Sample preparation and chemical and isotopic analysis*

158 *3.4.1 Feed samples*

159 Feed samples were homogenized prior to analysis using a Krups Speedy Pro homogenizer. Crude
160 protein was determined according to ISO⁽³⁹⁾, crude fat according to Bligh & Dyer⁽⁴⁰⁾, and dry matter
161 and ash according to Kolar⁽⁴¹⁾. Yttrium and phosphorus were determined according to ISO⁽⁴²⁾ and
162 DS⁽⁴³⁾, while amino acids were determined according to EC⁽⁴⁴⁾ and ISO⁽⁴⁵⁾. Starch analyses were
163 carried out according to the method by Bach Knudsen⁽⁴⁶⁾. Aliquots of the homogenized feed
164 samples were lyophilized and finely ground using mortar and pestle prior to determination of ¹³C
165 isotope enrichment and elemental carbon.

166 *3.4.2 Faecal samples*

167 Faecal samples were freeze dried prior to analysis using a Christ Beta 2-16 freeze dryer (Martin
168 Christ Gefriertrocknungsanlagen GmbH, Germany). Faecal protein was determined by elemental
169 analysis, according to the method described in section 3.4.4, assuming that protein equals

170 6.25×nitrogen. Faecal lipid was determined according to Bligh & Dyer⁽⁴⁰⁾. Faecal starch was
171 determined using a BioVision Starch assay kit (cat. #K647-100, Tecan GENios micro plate reader
172 (Austria) fitted with a 570 nm colorimetric filter), and yttrium was determined according to ISO⁽⁴²⁾
173 and DS⁽⁴³⁾. Aliquots of the faeces samples were lyophilized and finely ground using mortar and
174 pestle prior to determination of ¹³C isotope enrichment, and elemental carbon and nitrogen.

175 3.4.3 Fish samples

176 Fish sampled initially (1×12 samples) and by the end of each feeding period (3×12 samples) were
177 partly thawed and fractionated into livers, viscera (excluding liver), and carcass. Tissues were kept
178 at or below 0°C during fractionation in order to avoid enzymatic degradation of glycogen, and the
179 different fractions were immediately re-frozen at -20°C prior to further analysis. Liver samples were
180 homogenized in a frozen state using a mortar and pestle, and frozen carcass and viscera fractions
181 were pooled on tank level and homogenized for 60 seconds using a Tecator 1094 homogenizer
182 (Tecator AB, Höganäs, Sweden). An aliquot of each sample was further homogenized for 30
183 seconds using a Büchi Mixer B-400 (BÜCHI Labortechnik AG, Switzerland). The two fractions
184 (livers and carcass/viscera) were chemically and isotopically analysed separately according to the
185 procedures described below. In the following, the designation ‘whole fish’ and associated
186 metabolite pools refer to the summed whole body mass contribution of the two fractions (i.e., livers
187 and carcass/viscera) multiplied by their respective analytical values. Crude protein, crude fat, dry
188 matter and ash of the two fractions were determined as described for feed samples, while glycogen
189 analysis were determined using a BioVision glycogen assay kit (cat. #K646-100 / Tecan GENios
190 micro plate reader (Austria) fitted with a 570 nm colorimetric filter). Isolation of glycogen was done
191 according to Good *et al.*⁽⁴⁷⁾ Isolated glycogen samples were subsequently lyophilized prior to
192 determination of ¹³C isotope enrichment and elemental carbon. Lipid samples for ¹³C isotope
193 enrichment and elemental carbon analysis were obtained during the lipid extraction process of the
194 Bligh & Dyer procedure⁽⁴⁰⁾.

195 3.4.4 Isotopic analysis

196 Feed samples, isolated liver glycogen and lipid, and isolated carcass/viscera glycogen and lipid
197 samples were all analysed for ¹³C isotope enrichment and elemental carbon, while faecal samples
198 were analysed for ¹³C isotope enrichment and elemental carbon and nitrogen. Prior to isotopic
199 analysis aliquots of all samples were packed and weighed into tin capsules (standard weight pressed
200 tin capsules 5×3.5mm, cat# D1002, Elemental Microanalysis Ltd., Okehampton, UK) using an
201 analytical microbalance (Mettler Toledo MT5, Zürich, Switzerland). All carbon isotope and
202 elemental carbon and nitrogen analyses were carried out using a Thermoquest EA1110 CHNS-O

203 elemental analyser coupled to a Thermo Scientific Delta V advantage isotope ratio mass
204 spectrometer (IRMS) *via* a Thermo Scientific ConFlo IV module.

205 3.5 Calculations

206 Stable ^{13}C isotope enrichment ($\delta^{13}\text{C}$, ‰) of samples was calculated as:

207 $\delta^{13}\text{C} = ((R_{\text{sample}} - R_{\text{standard}}) / (R_{\text{standard}})) \times 1000$, where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample, and
208 R_{standard} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the reference standard calibrated against the international standard V-
209 PDB (Pee Dee Belemnite).

210 The ^{13}C atom percent excess (APE, ‰) of the samples was determined as the difference between the
211 atom percentage (^{13}C atm‰) of the enriched sample and the un-enriched sample ('blank'),
212 according to:

213 $\text{APE} (^{13}\text{C}) = ^{13}\text{C atm}\%_{\text{sample}} - ^{13}\text{C atm}\%_{\text{blank}}$

214 Atomic percentages were calculated as:

215 $^{13}\text{C atm}\% = (100 \times \text{AR} \times ((\delta^{13}\text{C} / 1000) + 1)) / (1 + \text{AR} \times ((\delta^{13}\text{C} / 1000) + 1))$, where AR is the
216 absolute $^{13}\text{C}/^{12}\text{C}$ ratio of V-PDB (0.0112372) as given by Craig⁽⁴⁸⁾.

217 Deposition rate of lipid derived from starch (LDR, mg/kg biomass/day) was calculated as:

218 $\text{LDR} = ((\text{mg lipid synthesized from starch } (t_i - t_0) / \text{kg biomass } (t_i)) / t_i - t_0)$, where mg lipid
219 synthesized from starch = (g carbon of starch origin found in whole body lipid fraction (t_i) / %
220 carbon in whole body lipid fraction (t_i)) \times 1000.

221 The carbon content of the whole body lipid fraction was determined from the mass spectrometry
222 analysis, while the amount of carbon deriving from starch found in the lipid fraction of the fish was
223 determined as:

224 $\text{g lipid C}_{(\text{starch origin})} = (\text{g lipid } ^{13}\text{C in excess} / \% ^{13}\text{C enrichment level of dietary starch})$, where lipid
225 $^{13}\text{C in excess} = (\text{g biomass } (t_i) \times \% \text{ whole fish lipid content } (t_i) \times \% \text{ carbon in whole fish lipid } (t_i) \times$
226 $\% \text{APE}_{\text{lipid}} (t_i))$, and $^{13}\text{C enrichment level of dietary starch} = (\% \text{APE}_{\text{diet}} / ((\% \text{dietary starch content}$
227 $\times \% \text{ carbon content of dietary starch}) / \% \text{ carbon in feed}))$.

228 Recovery of starch carbon in whole fish lipid fraction (RSCL, %) was calculated as:

229 $\text{RSCL} = \text{g lipid C}_{(\text{starch origin})} / \text{g digestible starch carbon intake}$.

230 3.6 Statistical analysis

231 Data on specific growth rates (SGR), feed conversion ratios (FCR), FI, hepatosomatic indices
232 (HSI), viscerosomatic indices (VSI), ADCs, and the chemical composition of whole fish and livers
233 were subjected to one-way ANOVA analysis, assessing significant differences among means using
234 Duncan's multiple range test⁽⁴⁹⁾. A t-test was used for testing differences between means in the ADC
235 of the two carbon isotopes. A probability of $P < 0.05$ was considered significant in all analyses. Data
236 on deposition rate of lipid derived from starch (LDR) and recovery of starch carbon in the lipid

fraction (RSCL) of whole fish were subjected to two-way ANOVA analysis to test for main effects of, and interactions between, diet and isotopic enrichment period, respectively. Significant differences found in main effect (when not interacting with other main effect) were subsequently assessed using Duncan's multiple range test. Regression analysis of temporal increase in APEs in liver lipid and glycogen and in whole fish glycogen pools was performed using a two-parameter exponential rise to maximum equation: $y = a \times (1 - \exp(-b \times x))$, where y is the ^{13}C APE, x is enrichment time, and a and b are constants. Regression analysis of the temporal increase in whole body lipid APEs gave a better fit when employing a linear regression: $y = b + a \times x$, where y is the ^{13}C APE, x is enrichment time, and a and b are constants. All statistical analyses were performed using the WinSTAT[®] for Microsoft[®] Excel, version 2009.1 software package (R. Fitch Software, Bad Krozingen, Germany).

4. Results

4.1 Diets and dietary ^{13}C enrichment

The composition and chemical analysis of the experimental diets are shown in table 1. The crude protein content of the diets was very similar (458-462 g/kg). The lipid content decreased from 218 g/kg in diet A to 139 g/kg in diet D, and the crude starch content increased from 54 g/kg in diet A to 236 g/kg in diet D. Data from the stable isotope analyses are presented in table 2. The dietary carbon content ranged from 45.2 to 47.9%. The $\delta^{13}\text{C}$ values of the non-enriched diets ranged between -21.7 and -21.5‰, while $\delta^{13}\text{C}$ values of the enriched diets were measured as 16.4, 48.2, 87.8 and 113.9‰ for diet A, B, C and D, corresponding to ^{13}C APEs of 0.04, 0.08, 0.12 and 0.15%, respectively. The measured ^{13}C enrichment of starch carbon was 0.83, 0.67, 0.70 and 0.64% for diets A, B, C and D, respectively.

4.2 ^{13}C enrichment of whole fish and livers

Isotope enrichment of the liver glycogen and liver lipid pools, and of the whole body glycogen and lipid pools, are presented in figure 1, while the associated regression equations are presented in table 3. The regression equations fitted the data very well with coefficients of determination (r^2) ranging between 0.828 and 1.000. The curves in figure 1: i-iii display saturation kinetics, moving asymptotically towards a value deduced from the equation constant a , (hereafter referred to as $\text{APE}_{\text{lim}} (t \rightarrow \infty)$). The theoretical maximum enrichment (TME) value any given $\text{APE}_{\text{lim}} (t \rightarrow \infty)$ could possibly approach equalled the ^{13}C enrichment of the starch fraction of the corresponding diet (table 3), and would only occur if dietary starch was the sole carbon contributor to that metabolite pool. Also, deduced from these equations, the theoretical time in days it would take APEs to reach 95% of their respective $\text{APE}_{\text{lim}} (t \rightarrow \infty)$ was designated $t_{95\%}$ (table 3) and indicated how fast the ^{13}C

270 enrichment of a given metabolite pool reached an equilibrium, which in turn could indicated the
271 turnover of that metabolite pool (i.e. low $t_{95\%}$ values indicated high turnover and high $t_{95\%}$ indicated
272 low turnover). Liver glycogen ^{13}C APE values rose exponentially toward an $\text{APE}_{\text{lim}} (t \rightarrow \infty)$ of
273 0.226, 0.343, 0.435 and 0.441% (figure 1: i), corresponding to 27.2, 51.0, 62.3 and 68.8% of the
274 TME for fish fed diets A, B, C and D, respectively. Furthermore, the regression analysis predicted
275 that fish fed diets A, B, C and D would reach $t_{95\%}$ in 67, 32, 20 and 19 days, respectively of feeding
276 on the enriched diets.

277 Liver lipid ^{13}C APE values rose exponentially towards $\text{APE}_{\text{lim}} (t \rightarrow \infty)$ values of 0.005, 0.021, 0.060
278 and 0.107% (figure 1: ii), corresponding to 0.6, 3.1, 8.6 and 16.7% of the TME for fish fed diets A,
279 B, C and D, respectively. Regression analysis on liver lipid APEs predicted that fish fed diets A, B,
280 C and D would reach $t_{95\%}$ in 15, 44, 48 and 51 days, respectively of feeding on the enriched diets.

281 Whole body glycogen pool APEs increased exponentially towards 0.137, 0.172, 0.235 and 0.248%
282 (figure 1: iii), corresponding to 16.5, 25.6, 33.7 and 38.8% of the TME for fish fed diets A, B, C
283 and D, respectively. Regression analysis of whole body glycogen APEs predicted that fish fed diets
284 A, B, C and D would reach $t_{95\%}$ in 103, 23, 16 and 9 days, respectively of feeding on the enriched
285 diets.

286 Whole body lipid ^{13}C APE values all increased linearly with time fed on the enriched diets (figure
287 1: iv), and the rate of enrichment was directly related to the dietary starch content, ranging from
288 0.0029 (diet A) to 0.0179% (diet D) after 30 days of feeding.

289 *4.3 Feed intake, growth and feed conversion ratio*

290 Results on feed intake, feed conversion and growth are presented in table 4. The fish accepted all
291 diets well, and the accumulated feed waste during the three feeding periods was approximately
292 2.2% of the total amount of feed fed to each tank, irrespectively of diet. No mortality occurred
293 throughout the trial. No significant differences in SGR (ranging from 1.19 – 1.29% / d), FCR
294 (ranging from 1.03 – 1.09) and FI (ranging from 1.28 – 1.33% / d) were found among the dietary
295 treatments.

296 *4.4 Digestibility of major nutrients and carbon isotopes*

297 The ADCs of the diets, as derived from large fish, are shown in table 4. The protein ADCs ranged
298 from 84.8 (diet C) – 91.6% (diet D), with fish fed diet D having a significantly higher protein
299 digestibility than fish fed the remaining diets. Lipid ADCs ranged from 87.0% in diet A to 90.7% in
300 diet C with no significant differences between dietary treatment groups. The apparent digestibility
301 of starch was significantly higher in fish fed diets A and B (99.3 and 99.6%, respectively) than in
302 fish fed diets C and D (97.3 and 96.5%, respectively). There was no difference in the apparent
303 digestibility of the two carbon isotopes (68.2 and 69.2% for ^{12}C and ^{13}C , respectively), determined

304 in the enriched diet A. Based on the study of Windell *et al.*⁽⁵⁰⁾ it was assumed that the ADCs derived
305 from larger fish applied to the smaller fish from this trial as well.

306 *4.5 Chemical composition of whole fish and livers*

307 The chemical composition of whole fish and livers at the end of the experiment is presented in table
308 5. In whole fish no significant effects of dietary treatment was seen in the content of crude protein,
309 crude lipid, dry matter, or ash. In contrast, the whole body glycogen content differed significantly
310 between all dietary treatments, equalling 1.8, 2.5, 3.4 and 4.3 g/kg for fish fed diet A, B, C and D,
311 respectively. There were no significant differences in the content of crude lipid, dry matter, or ash
312 in the liver. The liver protein content was inversely related to the dietary starch content, ranging
313 from 144.4 g/kg in fish fed diet A to 109.5 g/kg in fish fed diet D. Fish fed diets A and B differed
314 significantly from each other and from fish fed diets C and D. The liver glycogen content increased
315 with an increasing dietary starch content, being significantly lower in fish fed diet A (59.0 g/kg)
316 than in fish fed diets C and D (98.8 and 112.6 g/kg, respectively), while fish fed diet B (77.9 g/kg)
317 had a significantly lower liver glycogen content than fish fed diet D.

318 *4.6 Hepatosomatic and viscerosomatic indices*

319 The hepatosomatic index ranged from 1.1 - 1.7%, increasing significantly from diets A and B to
320 diets C and D, respectively. There were no significant differences in the viscerosomatic index
321 between dietary treatments (table 5).

322 *4.7 Recovery of carbon originating from starch in whole body lipid*

323 The percentage of carbon from dietary starch recovered in the lipid pool of whole fish (RSCL) is
324 presented in figure 2. The two-way ANOVA indicate that RSCL was significantly affected by
325 dietary treatment ($p<0.0001$), but not by the duration of the dietary enrichment period ($p=0.598$). A
326 multiple comparison analysis subsequently showed that RSCL was significantly lower in fish fed
327 diet B (4.3%) than fish fed diet C (5.7%) and D (8.0%). Furthermore, RSCL was significantly lower
328 in fish fed diet A (5.1%) than in fish fed diet D, while fish fed diet C had significantly lower RSCL
329 than fish fed diet D.

330 *4.8 Deposition rate of lipid synthesized from starch*

331 The deposition rate of lipid synthesized from starch (LDR) is presented in figure 3. The LDR
332 ranged from 18.7 – 123.7 mg/kg biomass/d in fish fed diet A and D, respectively. The two-way
333 ANOVA analysis shows that LDR was significantly affected by dietary treatment ($p<0.0001$), but
334 not by the duration of the dietary enrichment period ($p=0.557$). The subsequent multiple
335 comparisons analysis between dietary treatments showed an increase in LDR with increasing
336 dietary starch with all dietary treatment groups being significantly different.

337

338 5. Discussion

339 The purpose of the present study was to quantify the magnitude of *de novo* lipogenesis and
340 glycogenesis from dietary starch, when increasing the proportion of DE supplied by starch in diets
341 that were otherwise similar in DP and DE. A secondary objective was to determine the overall
342 importance of dietary starch in glycogen and lipid metabolism.

343 Simple, high quality raw material matrices were applied to assure highest possible nutrient quality
344 and avoid possible undesired effects on feed intake, growth performance and nutrient utilization of
345 anti-nutrients associated with certain vegetable raw materials⁽³⁾. Furthermore, the DP/DE ratio of the
346 diets was close to what is considered optimal for feed efficiency and nutrient retention⁽⁵¹⁾, and the
347 essential amino acid composition complied with general recommendations for this species^(52, 53).

348 The study clearly demonstrated both *de novo* lipogenesis and glycogenesis from dietary starch
349 (regardless of inclusion level), as evident from the continuous increase in ¹³C APE of glycogen and
350 lipid pools of both liver and whole fish tissues (figure 1: i-iv).

351 Of the four metabolite pools analysed, the liver glycogen pool displayed the highest enrichment
352 response, with APE_{lim} ($t \rightarrow \infty$) values increasing from 27.2 (diet A) to 68.8% (diet D) of their
353 respective TMEs and t_{95%} values decreasing from 76 to 19 days with increasing starch levels. The
354 magnitude of the ¹³C APE response, as well as the relatively low t_{95%} values observed in this
355 metabolite pool, confirms a rapid and efficient cascade of metabolic events leading to glycogenesis.
356 Furthermore, the decrease in t_{95%} associated with an increasing dietary starch inclusion level
357 indicates that turnover in this metabolite pool was stimulated by dietary starch. These results
358 support the general perception that both starch digestion⁽¹⁶⁻¹⁸⁾ and the following liver glucose
359 phosphorylation^(17, 19, 20, 27, 29) (glucose → glucose-6-phosphate (G6P)) are very efficient in gilthead
360 sea bream, even at high dietary starch inclusion levels. The observation that the regression curves
361 for diets C and D (figure 1: i) almost coincided may indicate a near maximum liver glycogen
362 synthesis from dietary starch when including 18 – 24% starch in the diet.

363 The enrichment of the whole body glycogen pool showed APE_{lim} ($t \rightarrow \infty$) values increasing from
364 16.5 – 38.8% of their respective TMEs, while t_{95%} values decreased from 103 to 9 days when
365 feeding diets A to D, respectively. Hence, a very rapid whole body glycogen turnover (t_{95%} = 9
366 days) is apparent at high dietary starch levels (diet D). Interestingly, almost two thirds of the whole
367 body glycogen and approximately one third of the liver glycogen must have originated from sources
368 other than dietary starch, even when feeding the high starch diet (diet D). Similar results were
369 obtained by Viegas *et al.* for European seabass, finding that as much as 98% of the blood glucose
370 production could be attributed to gluconeogenesis in both fasted and fed fish⁽³⁶⁾. The liver glycogen
371 content was much higher than the whole body glycogen content (table 5). Combined with the ¹³C
372 APE enrichment kinetics in the two pools (figure 1: i and iii) it therefore appears that hepatically

373 synthesized glycogen contributed significantly to the whole body glycogen pool, which in addition
374 must have received significant amounts of glycogen from other metabolic processes. Such results
375 might be explained by an apparent lack of gluconeogenesis regulation by dietary carbohydrates in
376 gilthead sea bream, expressed as fructose-1,6-biphosphatase (FBPase) and glucose-6-phosphatase
377 (G6Pase) activities, as described earlier^(17, 19, 20, 24, 27). Combined with glycogenesis from dietary
378 carbohydrates this appears to produce glycogen at rates that exceed the glycolytic capacity of the
379 fish, causing increases in both HSI and liver glycogen content as observed in the present and in
380 previous studies when feeding high carbohydrate diets^(14, 56, 61).

381 In contrast to the liver and whole body glycogen pools the $t_{95\%}$ of the liver lipid pool appeared to be
382 directly related to dietary starch inclusion level, indicating a reduction of turnover with increasing
383 dietary starch level. However, considering the very weak ^{13}C APE response and relatively low r^2 of
384 the regression for fish fed diet A, the associated $t_{95\%}$ value (i.e., 15 days) should probably be
385 disregarded, leaving the $t_{95\%}$ range between 44 to 51 days for fish fed diet B and D, respectively.

386 Similarly to the whole body glycogen pool, hepatically synthesized lipid from starch seemed to be
387 directed towards the whole body lipid pool. This was apparent from the approximately 5-fold higher
388 ^{13}C APE found in the liver lipid pool compared to the whole body lipid pool, and the linear
389 enrichment kinetics seen in whole body lipid, which indicate a passive storage function in the latter.

390 After 30 days of dietary ^{13}C starch enrichment, a maximum of 2.8% of the whole body lipid carbon
391 pool was of starch origin (calculated from the regression coefficients given in table 3),
392 corresponding to approximately 8.7% of the total whole body lipid deposited during the 30 days.

393 Thus, a relatively small proportion of the whole body lipid deposition could be attributed to
394 lipogenesis from dietary starch even at high dietary carbohydrate levels. This is also apparent when
395 considering the low efficiency (4.2 – 8.4%) with which starch carbon was recovered in the whole
396 body lipid fraction of the fish (RSCL; figure 2). Hence, more than 90% of the digested starch must
397 have been used for purposes other than lipogenesis regardless of dietary treatment. Interestingly, the
398 RSCL seemed to increase when increasing the dietary starch content (figure 2), meaning that the
399 actual amount of starch carbon recovered in the lipid fraction of fish fed diet D compared to fish fed
400 diet A was approximately 8-fold higher. This is also apparent from figure 3, which shows that the
401 deposition rate of lipid (LDR) originating from starch increased from 18.7 to 123.7 mg/kg
402 biomass/day when dietary starch increased from 6 to 24%. This confirms that *de novo* lipogenesis
403 from dietary carbohydrates was subject to regulation based on the dietary carbohydrate level,
404 consistent with earlier studies which found a strong correlation between dietary carbohydrate level
405 and enzyme activity related to lipogenesis^(24, 28, 54-56) (i.e. the activity of glucose-6-phosphate
406 dehydrogenase (G6PD)). Despite the fact that *de novo* lipogenesis from dietary carbohydrates was
407 indeed active and apparently subject to nutritional control, the contribution to the overall lipid

408 deposition appeared to have been modest in the present study. Hence, figure 4 presents lipid
 409 budgets of the four dietary treatment groups. The figure shows that the maximum contribution to
 410 lipid deposition from dietary starch was 12.3 grams of lipid out of a total deposition of
 411 approximately 150 grams when gaining one kg of biomass (fish fed diet D). The figure also shows
 412 that when feeding the highest starch levels (diet D), the total lipid deposition exceeded the sum of
 413 digested lipid and deposition from *de novo* synthesized lipid from starch. Thus, fish fed diet D
 414 would not even theoretically have been able to cover their total lipid deposition from dietary lipid
 415 and starch sources combined. Consequently, this lipid ‘deficit’ must have originated from *de novo*
 416 lipid synthesis presumably using protein as a carbon donor. This agrees with the stimulating effect
 417 of excess dietary protein on G6PD activity found by Enes *et al.*⁽¹⁷⁾ and the inherently poor protein
 418 retention reported for this species compared to species like salmonids.

419 The overall growth and feed performance results, as well as the daily feed intake were not
 420 significantly affected by dietary treatment, despite the fact that dietary DE increased by 1 MJ/kg
 421 ($\approx 5.6\%$) from diet A (17.9 MJ/kg) to diet D (18.9 MJ/kg). As increasing levels of dietary starch has
 422 been shown to negatively affect feed efficiency⁽²¹⁾, this may have masked any effects the slightly
 423 increased DE levels may have had on performance results in the present study. In addition, the
 424 growth period (30 days of feeding, corresponding to approximately 45% biomass increase) may
 425 have been too short to demonstrate significant differences in these parameters.

426 Fish fed diet D showed significantly better protein ADC than fish fed the remaining diets, which
 427 might have been an effect of the lower dietary cellulose content of this diet, although negative
 428 effects of dietary cellulose on protein digestibility have not been reported for other species fed diets
 429 with cellulose inclusion levels up to 20%⁽⁵⁷⁻⁵⁹⁾. The very high starch ADCs observed in this study
 430 (96.5 – 99.6%) and the inverse relationship between starch ADC and dietary starch level has been
 431 reported previously in both gilthead sea bream and European seabass^(16-18, 56). Ideally, analyses on
 432 dietary and faecal nutrient compositions used for ADC assessments should be carried out using
 433 identical protocols for both types of samples. However, the scarce amount of faecal matter available
 434 for analyses precluded use of the Kjeldahl method⁽³⁹⁾ for protein analysis and the Bach Knudsen
 435 method⁽⁴⁶⁾ for starch analysis on faecal samples. Thus, faecal starch content was determined using a
 436 BioVision starch assay kit and faecal protein was determined using elemental analysis.

437 Prior to the present study, the fish had formed part of a feeding trial, where they had been fed four
 438 diets similar to the experimental diets used here except for the addition of ^{13}C starch. Thus, fish had
 439 grown from an average weight of approximately 25 – 75 grams (un-labelled diets; pre-study) and
 440 further from 75 – 100 grams (labelled diets; present study), corresponding to a quadruplication of
 441 body weight on these diets. Despite of this, whole body protein, lipid, ash, and dry matter did not
 442 differ significantly among fish fed the four experimental diets. Thus, the increasing dietary starch

inclusion level did not affect the body composition, which is more or less in accordance with earlier findings⁽²¹⁾. Yet, some studies have found a positive relationship between dietary digestible carbohydrate and body lipid levels^(17, 24, 28), while others have found body lipid levels to be unaffected by both macro nutrient ratios in iso-DE diets⁽²¹⁾ and dietary energy level⁽⁶⁰⁾. Only whole body glycogen content was significantly elevated (from 1.8 to 4.3 g/kg) when increasing the dietary starch level. Liver lipid, ash, and dry matter were not significantly affected by dietary treatment, while liver protein decreased (from 144.4 – 109.5 g/kg) and liver glycogen increased (from 59.0 – 112.6 g/kg) in what resembled a 1:1 substitution of protein with glycogen when increasing dietary starch level. Also, HSI was significantly affected by the dietary treatment, increasing from 1.1 to 1.7% in fish fed diets A and D, respectively. Similar results have been reported in both gilthead sea bream and other species when increasing digestible dietary carbohydrate intake^(14, 56, 61).

6. Conclusion.

For the first time long term oral administration of ¹³C labelled starch has been applied to determine the role of dietary starch on glycogenesis and *de novo* lipogenesis in gilthead sea bream. The study corroborated the earlier reported high ADCs of processed/extruded starches⁽¹⁶⁻¹⁸⁾ followed by efficient glycogenesis. Also, endogenously produced glycogen from other metabolic processes (e.g. amino acid catabolism) seemed to contribute significantly to the total glycogen production of the fish regardless of dietary treatment. This could potentially be attributed to lack of dietary regulation of FBPase^(17, 19, 20, 24, 27), continuously diverting non-carbohydrate metabolites of the glycolytic pathway in the direction of glycogenesis. The combined endogenic and dietary contribution to glycogenesis appeared to have caused an accumulation of glycogen in both whole fish and liver tissues causing hepatomegaly in fish fed high starch diets. Also, *de novo* lipogenesis from dietary starch was proven active and under apparent nutritional control. However, a maximum of 8.4% of the digested starch carbon could be recovered in the lipid fraction of the fish, indicating that dietary starch seemed to play only a minor role in the overall lipid budget of gilthead sea bream. Considering the significant contribution of non-carbohydrate metabolites (most likely originating from protein catabolism) to glycogenesis, and the apparent ‘deficit’ in the lipid budget presented, the results strongly hint that dietary protein may play a major role not only for anabolic purposes, but also for short term (glycogen) and long term (lipid) energy storage purposes in gilthead sea bream, which in turn could explain the relatively poor protein retention typically found in this species.

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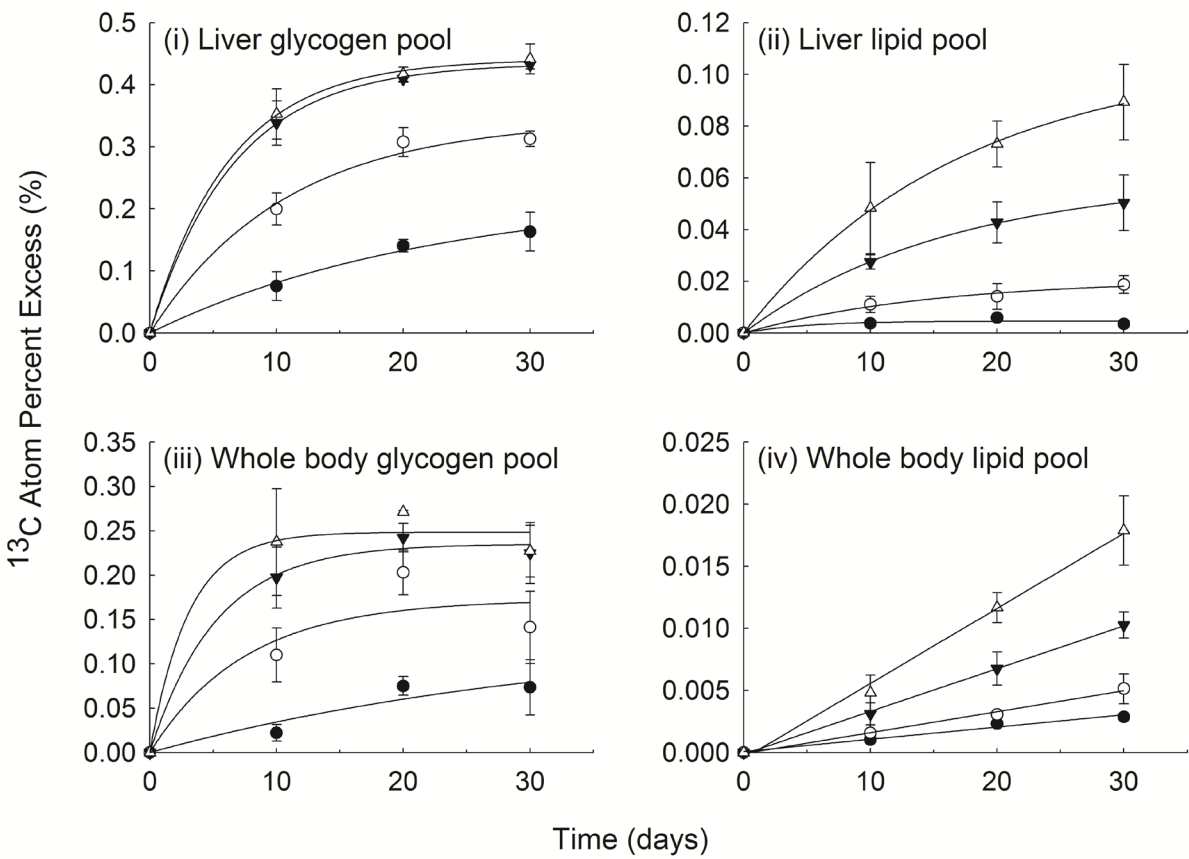
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Figure 1



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656 **Figure 1.** ^{13}C atom percent excess (APE (%), mean \pm SD, n = 3) measured in (i) liver glycogen
657 pool, (ii) liver lipid pool, (iii) whole body glycogen pool, and (iv) whole body lipid pool, measured
658 after 10, 20 and 30 days of dietary ^{13}C starch enrichment. Associated regression analyses are
659 presented in table 3. \bullet = diet A; \circ = diet B; \blacktriangledown = diet C; Δ = diet D

Figure 2

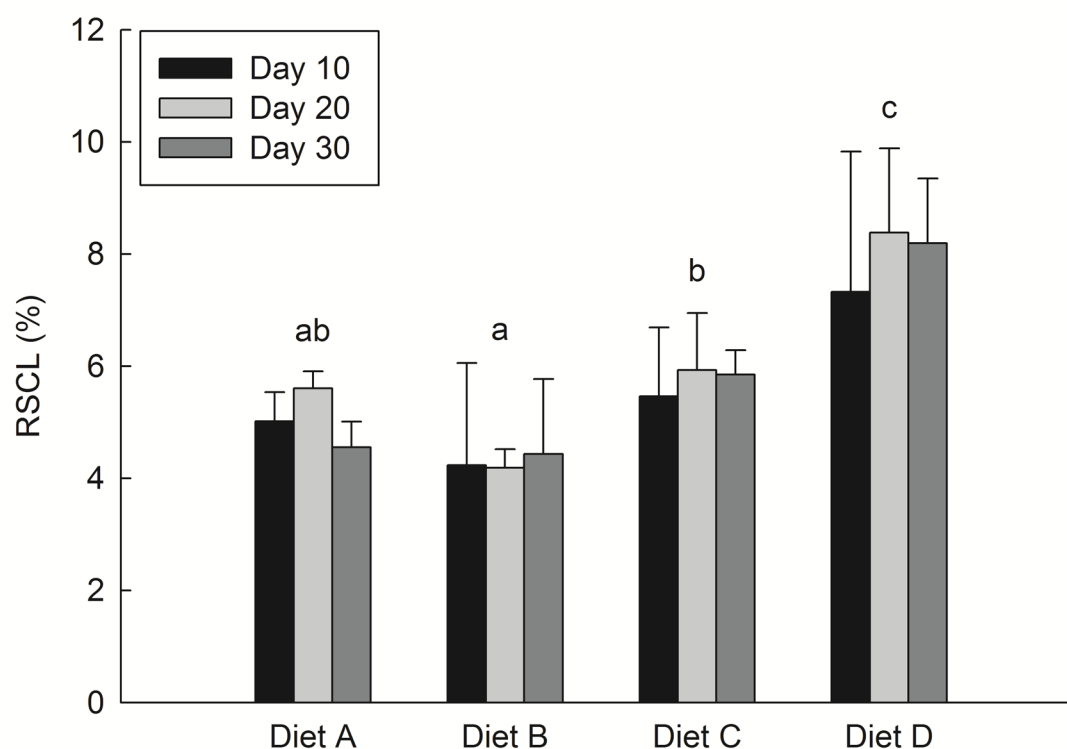


Figure 2. Percentage of the digested starch carbon recovered in lipid carbon fraction of whole fish (RSCL (%), mean \pm SD, n = 3) after being fed the enriched diets for 10, 20 and 30 days, respectively. Two-way ANOVA showed significant differences between dietary treatments ($p < 0.0001$), while there were no significant differences between enrichment periods ($p = 0.598$), and there were no interaction between dietary treatment and enrichment period ($p = 0.947$). Different letters above bars indicate significant difference among dietary treatments (Duncan's multiple range test, $p < 0.05$)

Figure 3

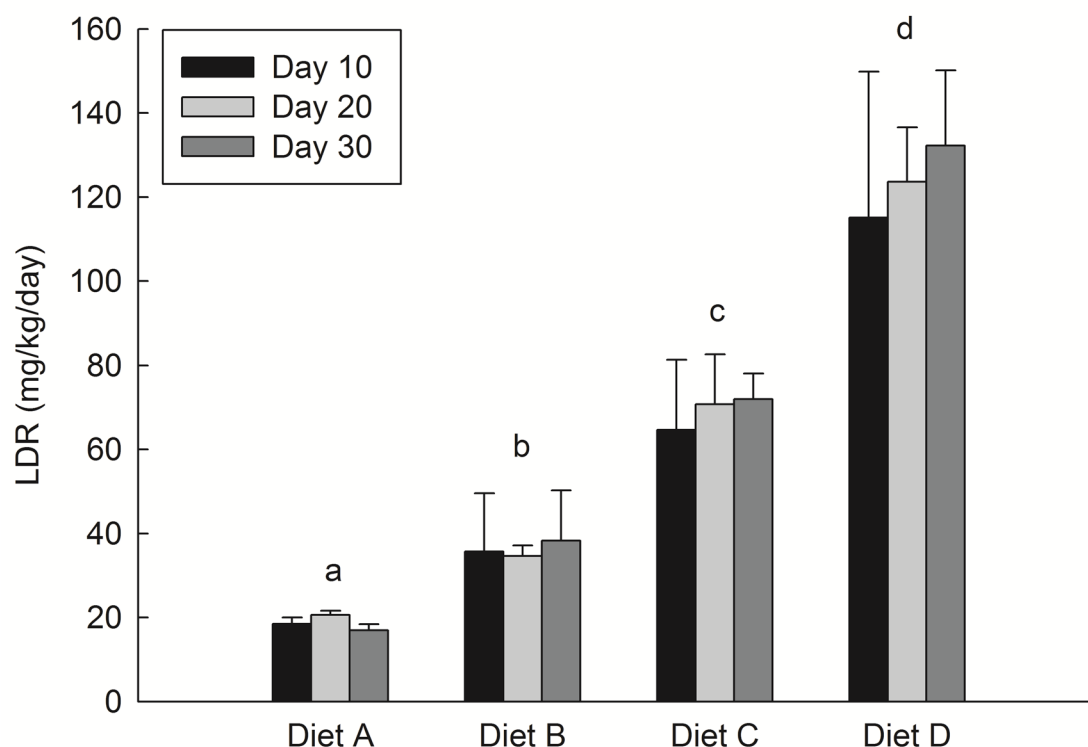


Figure 3. Deposition rate of lipid derived from starch (LDR, mg/kg/day, mean \pm SD, n = 3) in fish fed diets containing 6 (diet A), 12 (diet B), 18 (diet C) and 24% (diet D) starch over three, 10 days feeding periods. Two-way ANOVA showed significant differences between dietary treatments ($p < 0.0001$), while there were no significant differences between enrichment periods ($p = 0.557$), and no interaction between dietary treatment and enrichment period ($p = 0.950$). Different letters above bars indicate significant difference among dietary treatments (Duncan's multiple range test, $p < 0.05$)

Figure 4

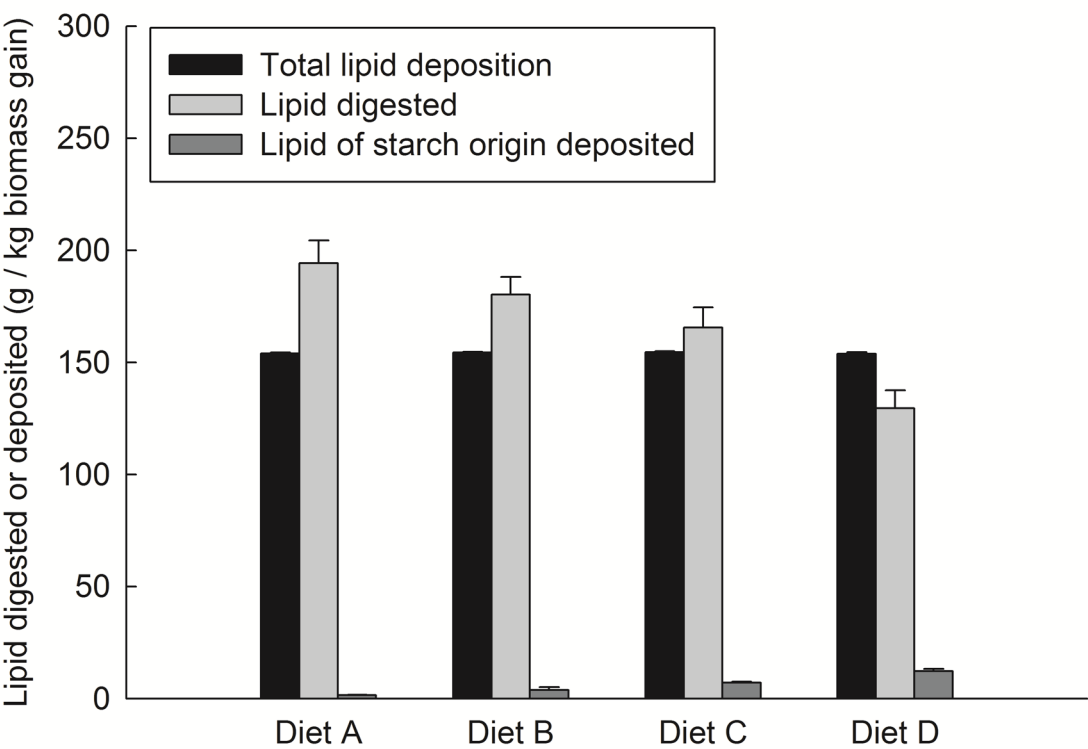


Figure 4. Lipid budget when accreting one kg of biomass (lipid deposited or digested, g, mean \pm SD, n = 3) in fish fed diets containing 6 (diet A), 12 (diet B), 18 (diet C) and 24% (diet D) starch.

712 **Table 1.** Diet formulation and chemical composition of experimental diets

Diet	A	B	C	D
Ingredients (g/kg)*				
Fish meal	607	607	607	608
Fish oil	152	127	103	78
Wheat starch	68	136	204	272
Cellulose	158	114	70	26
Guar gum	5	5	5	5
Vitamin and mineral mix	3	3	3	3
Monocalcium phosphate	6	6	6	6
¹³ C labelled starch†	0.3	0.6	0.9	1.2
Yttrium oxide	0.3	0.3	0.3	0.3
Proximate composition (g/kg)				
Protein	461	461	462	458
Fat	218	189	168	139
Starch	54	121	179	236
DM	964	952	956	951
Ash	80	80	81	80
Phosphorus	15	15	15	15
Essential amino acids (EAA; g/kg)				
Arg	25.2	25.2	25.1	25.0
His	11.6	11.5	11.4	11.4
Ile	19.6	19.6	19.4	19.5
Leu	34.5	34.4	34.3	33.8
Val	24.0	23.8	23.5	23.2

Lys	3.79	3.79	3.72	3.72
Met+Cys	19.2	18.9	18.9	18.5
Phe	18.3	18.4	18.2	18.1
Thr	21.2	20.9	20.8	20.3
Trp	5.6	5.6	5.7	5.5
Calculated dietary energies (MJ/kg)				
Gross energy†‡	23.1	22.3	21.9	21.1
Digestible energy§	17.9	18.2	18.4	18.9

*Fish meal: TripleNine Fish Protein, Esbjerg, Denmark; Fish oil: South American (Peru); Wheat starch: Cargill, Belgium; Cellulose: Vitacel R200, Rettenmaier und Söhne GmbH, Germany; Guar gum: HV200, LCH A/S, Frederiksberg, Denmark; ¹³C labelled starch: Cambridge Isotope Laboratories Inc., Algal starch (U-¹³C, 98%+), cat. #CLM-1699-0, Andover, MA, USA; Vitamin and mineral mix is estimated to meet the requirements according to the U.S. National Research Council recommendations⁽⁶²⁾.

†Each of the four diets were produced in a ¹³C enriched version (adding ¹³C labeled starch) and a non-enriched version (adding wheat starch) in order to determine ¹³C APE of experimental diets

‡ Gross energy (GE, MJ/kg) was calculated as the sum of the dietary content of protein, lipid and nitrogen-free extract (NFE), multiplied by their respective energetic values upon complete oxidation⁽⁶³⁾: $GE = (P_{\text{diet}} \times 23.66) + (L_{\text{diet}} \times 39.57) + (NFE_{\text{diet}} \times 17.58)$, where P_{diet} , L_{diet} and NFE_{diet} refer to the dietary protein, lipid and NFE content (%), respectively. NFE was calculated as the sum of dietary protein, lipid, ash and water deducted from 100% (by difference).

§ The digestible energy (DE, MJ/kg) content was calculated as the dietary gross energy, but with the ADC of each nutrient multiplied into their respective terms:

$DE = (P_{\text{diet}} \times 23.66 \times ADC_{\text{protein}}) + (L_{\text{diet}} \times 39.57 \times ADC_{\text{lipid}}) + (S_{\text{diet}} \times 17.58 \times ADC_{\text{starch}})$, where S_{diet} is the dietary starch content, and ADC_{protein} , ADC_{lipid} and ADC_{starch} are the ADCs of protein, lipid and starch, respectively.

Table 2. Elemental carbon and stable carbon isotope analysis on the four experimental diets (mean \pm SD, n = 3)

Diet	A		B		C		D	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Carbon content of diet (%)								
Non-enriched diets	47.87	0.59	47.42	0.35	46.73	0.27	45.57	0.03
¹³ C-enriched diets	47.82	0.47	47.00	0.54	46.14	0.19	45.19	0.15
$\delta^{13}\text{C}$ values of diets (‰)								
Non-enriched diets	-21.50	0.04	-21.65	0.04	-21.59	0.13	-21.55	0.02
¹³ C-enriched diets	16.36	0.38	48.21	1.84	87.75	1.82	113.89	2.59
¹³ C atom percent excess (APE, %)								
¹³ C-enriched diets	0.0416	0.0004	0.0767	0.0020	0.1201	0.0021	0.1487	0.0028
¹³ C enr. of starch C (%)	0.831	0.006	0.672	0.013	0.698	0.012	0.641	0.011

753 **Table 3.** ¹³C APE regression equation coefficients and constants calculated from regression analysis
754 of liver glycogen and lipid pools and whole body glycogen and lipid pools.

Metabolite pool	Dietary treatment	a	b	r ²	APE _{lim} * (t→∞)	t _{95%} †	Enrich. (% of TME)‡
Liver glycogen pool							
	A	0.226	0.045	0.994	0.226%	67	27.2%
	B	0.343	0.094	0.993	0.343%	32	51.0%
	C	0.435	0.150	1.000	0.435%	20	62.3%
	D	0.441	0.159	1.000	0.441%	19	68.8%
Liver lipid pool							
	A	0.005	0.206	0.828	0.005%	15	0.6%
	B	0.021	0.068	0.985	0.021%	44	3.1%
	C	0.060	0.062	1.000	0.060%	48	8.6%
	D	0.107	0.059	0.999	0.107%	51	16.7%
Whole body glycogen pool							
	A	0.137	0.029	0.904	0.137%	103	16.5%
	B	0.172	0.133	0.867	0.172%	23	25.6%
	C	0.235	0.191	0.993	0.235%	16	33.7%
	D	0.248	0.327	0.979	0.248%	9	38.8%
Whole body lipid pool							
	A	1×10 ⁻⁴	8×10 ⁻⁵	0.966	n/a	n/a	n/a
	B	2×10 ⁻⁴	-9×10 ⁻⁵	0.991	n/a	n/a	n/a
	C	3×10 ⁻⁴	-1×10 ⁻⁴	0.999	n/a	n/a	n/a
	D	6×10 ⁻⁴	-5×10 ⁻⁴	0.993	n/a	n/a	n/a

755 *APE_{lim} (t→∞) refers to the ¹³C APE value asymptotically approached by a metabolite pool when
756 enrichment time goes towards infinity.

757 † $t_{95\%}$ refers to the theoretical time (in days) it would take APE of a metabolite pool to reach 95% of
758 $APE_{lim}(t \rightarrow \infty)$. Calculated as: $t_{95\%} = \ln(0.05) / -b$, where b is the equation constant as given in the
759 table.

760 ‡TME refers to the theoretical maximum ^{13}C enrichment value any given $APE_{lim}(t \rightarrow \infty)$ could
761 possibly approach, i.e., equalling the ^{13}C enrichment of the starch fraction of the corresponding diet.

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Table 4. Specific growth rate, feed conversion ratio, daily feed intake and apparent digestibility coefficients of major nutrients and stable carbon isotopes (mean \pm SD, df=3)^{*}

Diet	A		B		C		D		p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Growth, feed conversion ratios and feed intake									
SGR (%/d) [†]	1.25	0.05	1.22	0.04	1.19	0.06	1.29	0.10	0.37
FCR (kg/kg) [‡]	1.03	0.05	1.07	0.05	1.09	0.06	1.03	0.06	0.48
FI (%/d) [§]	1.28	0.01	1.31	0.02	1.30	0.02	1.33	0.03	0.11
Apparent digestibility coefficients of major nutrients (ADC; %)									
Protein	86.7 ^a	0.1	86.6 ^a	2.3	84.8 ^a	0.4	91.6 ^b	0.3	0.001
Lipid	87.0	3.1	89.0	0.8	90.7	2.2	90.5	3.7	0.353
Starch	99.3 ^a	0.2	99.6 ^a	0.1	97.3 ^b	0.3	96.5 ^b	0.9	<0.001
Apparent digestibility coefficients of carbon isotopes (ADC, %)									
¹² C	68.2	1.2	n/a		n/a		n/a		0.363 [¶]
¹³ C	69.2	1.1	n/a		n/a		n/a		

^{*}Mean values within a row not sharing the same superscript letter were significantly different (Duncan's multiple range test, P<0.05).

[†]The specific growth rate calculated according to Hopkins (1992)⁽⁶⁴⁾ as: $SGR = \ln(\text{biomass}_{\text{end}}) / \text{biomass}_{\text{initial}} / (\text{days in trial}) \times 100$

[‡]The feed conversion ratio: $FCR = \text{feed consumed} / \text{biomass gained}$

[§]Daily feed intake: $FI = \text{feed consumed} / \text{expected biomass}$

|The ADC of nutrient X: $ADC_{(X)} = 1 - ((I_{\text{diet}} \times X_{\text{faeces}}) / (I_{\text{faeces}} \times X_{\text{diet}}))$, where I_{diet} and I_{faeces} is the yttrium concentration recovered in the diet and faeces, respectively, and X_{faeces} and X_{diet} is the concentration of X (protein, lipid, starch or carbon isotope) recovered in the faeces and diet, respectively⁽⁶⁵⁾.

[¶] p-value is based on a t-test comparing the ADC's of the two carbon isotopes.

804 **Table 5.** Chemical composition of whole fish and livers (mean ± SD, df=3) *

Diet	A		B		C		D		p-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Chemical composition of whole fish (g/kg)									
Crude protein	173.9	1.9	170.4	1.3	174.9	3.3	175.8	4.0	0.172
Crude lipid	147.4	5.7	148.9	11.0	147.0	4.0	142.5	2.9	0.684
Dry matter	345.7	5.9	346.8	8.7	351.6	1.0	351.0	4.9	0.537
Ash	37.3	1.3	38.0	1.3	38.0	1.0	40.0	0.6	0.070
Glycogen	1.8 ^a	0.1	2.5 ^b	0.2	3.4 ^c	0.1	4.3 ^d	0.4	<0.001
Chemical composition of livers (g/kg)									
Crude protein	144.4 ^a	4.2	127.4 ^b	4.2	116.4 ^c	4.2	109.5 ^c	1.1	<0.001
Crude lipid	122.1	23.0	126.1	32.3	115.6	22.9	119.7	18.2	0.960
Dry matter	328.4	21.7	375.5	83.0	334.9	1.8	341.5	5.9	0.571
Ash	15.8	3.0	12.4	1.7	12.4	1.0	11.5	0.8	0.099
Glycogen	59.0 ^a	9.2	77.9 ^{ab}	20.1	98.8 ^{bc}	10.1	112.6 ^c	9.4	0.004
Hepatosomatic and viscerosomatic indices (%)									
HSI†	1.1 ^a	0.2	1.2 ^a	0.4	1.6 ^b	0.4	1.7 ^b	0.4	<0.001
VSI‡	6.8	0.7	6.7	1.1	6.9	0.9	6.5	0.7	0.298

805 * Mean values within a row not sharing the same superscript letter were significantly different
806 (Duncan's multiple range test, P<0.05).

807 † The hepatosomatic index: $HSI = (\text{weight}_{\text{liver}} / \text{weight}_{\text{whole body}}) \times 100$

808 ‡ The viscerosomatic index: $VSI = (\text{weight}_{\text{viscera}} / \text{weight}_{\text{whole body}}) \times 100$

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3. Paper II: *De novo* lipid synthesis from dietary protein in gilthead sea bream (*Sparus aurata*) quantified with stable isotopes – effects of DP/DE ratio

***De novo* lipid synthesis from dietary protein in gilthead sea bream (*Sparus aurata*) quantified
with stable isotopes - effects of DP/DE ratio**

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Running title: Lipogenesis in gilthead sea bream

Keywords: gilthead sea bream: stable isotopes: DP/DE ratio: lipogenesis

1. Abstract

The effects of varying dietary digestible protein (DP) and digestible energy (DE) content on performance, nutrient retention efficiency and *de novo* lipogenesis of DP origin was examined in triplicate groups of gilthead sea bream (*Sparus aurata*), fed nine extruded experimental diets. In order to trace the metabolic fate of dietary protein, 1.8% was replaced with isotope labelled whole protein (>98% ^{13}C). The experiment was divided into a growth period lasting 89 days, growing fish from approximately 140 to 350 g, followed by a three day period feeding isotope enriched diets. Isotope ratio mass spectrometry was applied to quantify ^{13}C enrichment of whole body lipid from dietary DP. Between 18.6 and 22.4% of the carbon derived from protein was recovered in the lipid fraction of the fish, and between 21.6 and 30.3% of the total lipid deposited could be attributed to dietary protein. Digestible protein retention (DPR) was significantly improved by reductions in dietary DP/DE ratio, while the opposite was true for apparent digestible lipid retention (aDLR). Both overall digestible energy retention (DER) and whole body proximate composition of whole fish were largely un-affected by dietary treatments, while feed conversion ratios were significantly improved with increasing dietary energy density. The study suggests that gilthead sea bream efficiently utilizes dietary nutrients over a wide range DP/DE ratios and energy densities. In addition, they appear to endeavour a certain body energy status rather than maximising growth, which in the present trial was apparent from a inherently high *de novo* lipogenesis originating from DP.

2. Introduction

One of the biggest expenses in modern aquaculture is feed cost. Thus, maximized feed utilisation per unit cost is of paramount importance in maintaining an economically viable aquaculture enterprise. In commercial fish feed production the main concern is the quality, quantity and ratio between nutrients supplied through the raw materials used, and the cost of combining these to fit the nutritional requirements of a specific farmed species at a specific life-stage. These nutrient requirements, however, are not absolute. Rather, they should be present in the correct proportion to each other as pointed out by Wilson⁽¹⁾ with respect to protein requirements. According to him, the protein requirement of an animal comprises a well-balanced mixture of essential and non-essential amino acids, where protein digestibility, amino acid profile and energy concentration of the diet is also considered. Consequently, two of the most commonly used diet optimization “tools” in aquaculture comprise amino acid optimization (‘ideal protein concept’)⁽²⁻⁴⁾, and optimization of the ratio between digestible protein (DP) and digestible energy (DE)⁽⁵⁻⁹⁾. The optimal DP/DE ratio refers to the minimum amount of DP required for optimizing a certain production trait, such as growth,

64 feed conversion or protein retention at a given DE density. Diets containing DP in excess of
65 requirements will lead to excessive protein deamination, which in turn increases discharge of
66 nitrogenous compounds into the environment^(10, 11). Additionally, protein is the most costly macro
67 nutrient in aquaculture diets. Thus, there is an economic incentive not to include this nutrient in
68 excess of requirements.

69 Historically, gilthead sea bream (*Sparus aurata*) has been perceived to have a high dietary protein
70 requirement, and relatively poor protein utilization and feed conversion compared to other
71 aquacultured species such as salmonids. This is also reflected in the reported optimal DP/DE ratios
72 for this species^(12, 13), which are considerably higher than for farmed salmonids at the same life-
73 stage⁽¹⁴⁻¹⁶⁾. Irrespective of species, practically all DP/DE studies reported so far have focused on
74 optimizing protein retention. In practice this is typically done by reducing the dietary DP/DE level,
75 by substituting DE supplied from DP with DE supplied from non-protein DE sources such as fat<sup>(17-
76 20)</sup> or carbohydrates^(18, 21-25). Only a few studies have commented on the metabolic fate of non-
77 retained (deaminated) protein in this respect^(26, 27).

78 Recent studies have indicated that a substantial part of the deaminated amino acids in Blackspot
79 seabream (*Pagellus bogaraveo*) were converted into fatty acids *de novo*⁽²⁷⁾. This was expressed by
80 increased hepatic lipogenic enzyme activities and increased hepatic content of palmitic and stearic
81 acids, which are generally recognized to be the main products of *de novo* lipogenesis⁽²⁸⁾.
82 Additionally, studies by Enes^(29, 30) have shown a correlation between dietary protein level and
83 lipogenic enzyme activity both in gilthead sea bream and European seabass (*Dicentrarchus labrax*),
84 indicating that protein may contribute to lipid biosynthesis in this species. However, since
85 deaminated protein can precede both gluconeogenesis, lipogenesis and complete oxidation for
86 energy purposes it is hard to quantitatively conclude on the fate of deaminated protein.

87 The main purpose of the present study was to quantify the amount of dietary protein endogenously
88 converted to body lipid *de novo* in gilthead sea bream using nine diets enriched with stable isotopes,
89 that differed in DP/DE level and energy density. Additionally, macro nutrient retention efficiencies,
90 growth and feed performance parameters were determined. To achieve this, a study comprising two
91 trial periods was conducted. Firstly, a 89 days growth period was carried out feeding gilthead sea
92 bream nine diets differing in DP and DE content. Based on this growth period the growth (SGR),
93 feed conversion ratio (FCR), nutrient digestibility coefficients (ADC), digestible protein retention
94 (DPR), digestible energy retention (DER) and apparent lipid retention (aDLR) was determined.
95 Secondly, and immediately following the growth period, fish were fed their respective diets for
96 three more days, only now diets were added trace amounts of ¹³C labelled protein isolate. This was
97 done to determine the extent to which dietary protein was converted into body lipid endogenously,
98 and to determine how much this lipid biosynthesis contributed to the overall lipid deposition in the

99 fish. As deposited lipid could originate from both dietary and endogenous sources (*de novo*
100 lipogenesis), digestible lipid retention efficiencies are henceforth referred to as “apparent”.

101 **3. Materials and methods**

102 *3.1 Culture conditions and fish*

103 Gilthead sea bream with an average individual weight of approximately 120 grams were obtained
104 from a commercial fish farm (Ferme Marine de Douhet, Île d’Oléron, France). They were
105 subsequently stocked into a recirculated aquaculture system (RAS) comprising 27 fibre glass tanks
106 with a volume of 800 L each at a stocking density of 20 fish/tank (BioMar research facility, the
107 North Sea Research Centre, Hirtshals, Denmark). The tanks were fitted with a central bottom drain
108 designed to quickly and efficiently remove faeces and un-eaten feed pellets from the water by
109 means of externally mounted swirl separators. The trial facility was supplied with filtered North Sea
110 water with a salinity of 34 g/l, and temperature was kept at 24°C throughout the trials. Water quality
111 was monitored daily, maintaining O₂ saturation between 80-100%, NH⁴⁺ below 1.0 mg/l, NO²⁻
112 below 1.0 mg/l, and NO³⁻ below 100 mg/l. pH was adjusted to 7.0 using sodium bicarbonate when
113 necessary. Tanks were supplied with system water at a flow rate of 1200 l/tank/h. A 14 h light : 10 h
114 dark photoperiod was maintained throughout the trials. All fish were acclimated to the facility for 2
115 weeks during which they were fed a commercial diet (BioMar EFICO Sigma 860) according to
116 commercial feeding table value (1.5% of the biomass/d).

117 *3.2 Experimental diets*

118 Nine experimental diets were prepared using the Allix² feed formulation software (A-systems S.A.,
119 France) (Table 1). The main dietary ingredients were fish meal, fish oil, wheat and field peas, and
120 diets were formulated to contain 3 DP levels (330, 360 or 380 g/kg) and 3 DE levels (20, 21 or 22
121 MJ/kg) in a 3×3 factorial design. Diets were named according to their DP and DE content (LP, MP
122 or HP for low, medium or high DP content, respectively, and LE, ME or HE for low, medium or
123 high DE content, respectively). For example, diet LPLE refers to the low DP: low DE diet
124 (expected to contain 330 g/kg DP and 20 MJ/kg DE). Two versions of each diet were made: one
125 where approximately 1.8% of the dietary protein was substituted with ¹³C labelled (97-98% ¹³C)
126 protein isolate (Cambridge Isotope Laboratories, Inc., Andover, MA, USA); and one un-labelled
127 version. The diets were supplemented with a vitamin and mineral premix, as well as mono calcium
128 phosphate. Guar gum was added (5 g/kg) to enhance pellet stability and accurately quantify feed
129 waste, and yttrium oxide (Y₂O₃) was added (0.3 g/kg) as an inert marker enabling indirect
130 measurements of nutrient and stable isotope digestibility. Diets were prepared at the BioMar

131 TechCenter (Brande, Denmark) using a twin screw extruder (Clextal BC-45, Firminy, France) to
132 produce 4.5 mm pellets. Following extrusion, diets were dried in a six-level Geelen counter flow
133 continuous dryer (Geelen Counterflow, Haalen, The Netherlands), vacuum coated with fish oil, and
134 cooled.

135 3.3 Experimental procedures

136 The study comprised two trial periods: 1) a 89 days growth period (growing fish from approx. 140
137 to approx. 340 g) feeding each of the nine unlabelled diets to three replicate tanks (i.e. 27 tanks in
138 all), and concluded by a faeces stripping procedure to determine the apparent digestibility
139 coefficients of macro nutrients; and subsequently 2) a three day enrichment period feeding the ^{13}C
140 enriched versions of the experimental diets to determine the proportion of dietary protein converted
141 into body lipid *de novo*, and to determine the apparent digestibility coefficients of the two stable
142 carbon isotopes (^{12}C and ^{13}C). All procedures were carried out in accordance to EC directive
143 86/609/EEC for animal experiments⁽³¹⁾.

144 3.3.1 Growth period

145 Five randomly chosen fish were removed from each of the 27 tanks at start-up and euthanized using
146 250 mg/l tricaine methanesulfonate (MS-222). The 5×27 fish were subsequently pooled and stored
147 at -20°C until analysis, constituting the initial fish sample. The remaining 15 fish in each tank were
148 weighed, and the nine experimental diets were fed to triplicate tanks for 89 days. The fish were fed
149 a ration re-calculated from a commercial feeding table for gilthead sea bream, allowing a restrictive
150 iso-DE feeding regime based on the expected DE content of the respective experimental diets. Any
151 un-eaten feed was collected daily and subtracted in calculations of feed intake. Fish were fed from
152 08:00 to 14:00 h using automatic belt feeders. On day 89 the final meal was administered 18 h prior
153 to faeces stripping, where the fish were anaesthetised using MS-222 (50 mg/l), and a gentle bi-lateral
154 pressure was applied to the hindgut in order to provoke defecation. Faeces obtained from fish within
155 each tank was pooled and immediately frozen at -20°C . Twenty four hours after the stripping
156 procedure fish were bulk weighed and seven fish from each tank were removed, euthanized using
157 MS-222 (250 mg/l) and subsequently stored at -20°C for chemical and isotope analysis.

158 3.3.2 ^{13}C enrichment period

159 Following the stripping procedure of the growth trial the eight remaining fish in each tank were fed
160 their respective nine experimental diets for three more days, only now in the ^{13}C protein enriched
161 version at a feeding rate calculated as described in section 3.3.1. Un-eaten feed was collected daily
162 and subtracted in calculations of feed intake. 18 hours after the final meal, fish fed diet MPME (3

tanks in all) were stripped for faecal matter according to the method described in section 3.3.1. This was done in order to determine the ADC of the two stable carbon isotopes (^{12}C and ^{13}C), assuming that they were representative of all experimental diets. Fish were starved for 48 hours after the final meal and subsequently euthanized using MS-222 (250 mg/l), weighed and stored at -20°C for chemical and isotope analysis.

3.4 Sample preparation and chemical and isotopic analysis

3.4.1 Feed samples

Feed samples were homogenized prior to analysis using a Krups Speedy Pro homogenizer. Crude protein was determined according to ISO⁽³²⁾, crude fat according to Bligh & Dyer⁽³³⁾, and dry matter and ash according to Kolar⁽³⁴⁾. Yttrium was determined according to ISO⁽³⁵⁾ and DS⁽³⁶⁾. Starch analyses were carried out according to the method by Bach Knudsen⁽³⁷⁾, while amino acids were determined according to EC⁽³⁸⁾ and ISO⁽³⁹⁾. Aliquots of the homogenized feed samples were lyophilized and finely ground using mortar and pestle prior to determination of ^{13}C isotope enrichment and elemental carbon (see section 3.4.4).

3.4.2 Faecal samples

Faecal samples were freeze dried prior to analysis using a Christ Beta 2-16 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Faecal protein was determined by elemental analysis according to the method described in section 3.4.4, assuming that protein equals $6.25 \times \text{nitrogen}$. Faecal lipid was determined according to Bligh & Dyer⁽³³⁾. Faecal starch was determined using a BioVision Starch assay kit (cat. #K647-100, Tecan GENios micro plate reader (Austria) fitted with a 570 nm colorimetric filter), and yttrium was determined according to ISO⁽³⁵⁾ and DS⁽³⁶⁾. Aliquots of the faeces samples were lyophilized and finely ground using mortar and pestle prior to determination of ^{13}C isotope enrichment, and elemental carbon and nitrogen (see section 3.4.4).

3.4.3 Fish samples

Fish sampled initially (1 pooled sample), at the end of the growth trial (27 samples), and after the ^{13}C enrichment trial (27 samples) were homogenized in a two-step procedure prior to chemical and/or isotope analysis. Frozen fish samples were homogenized for 60 seconds using a Tecator 1094 homogenizer (Tecator AB, Höganäs, Sweden), and an aliquot of each sample was further homogenized for 30 seconds using a Büchi Mixer B-400 (BÜCHI Labortechnik AG, Switzerland). All sample aliquots were subjected to crude protein, crude lipid, dry matter and ash analyses, using

the same methodology as described for feed (section 3.4.1). Samples obtained at the end of the growth trial and at the end of the isotope enrichment trial were additionally subjected to stable carbon isotope analysis of their respective lipid fractions. Lipid samples for isotope analysis were obtained during the lipid extraction process of the Bligh & Dyer⁽³³⁾ procedure.

3.4.4 Isotopic analysis

Feed (enriched and un-enriched) and isolated whole body lipid samples were all subjected to stable isotope ($\delta^{13}\text{C}$) and elemental carbon analysis, while faecal samples were additionally subjected to elemental nitrogen analysis. Prior to isotopic analysis aliquots of all samples were packed and weighed into tin capsules (standard weight pressed tin capsules 5×3.5mm, cat# D1002, Elemental Microanalysis Ltd., Okehampton, UK) using an analytical microbalance (Mettler Toledo MT5, Zürich, Switzerland). All stable isotope enrichment, elemental carbon and nitrogen analyses were carried out using a Thermoquest EA1110 CHNS-O elemental analyser coupled to a Thermo Scientific Delta V advantage isotope ratio mass spectrometer (IRMS) *via* a Thermo Scientific ConFlo IV module.

3.5 Calculations

Stable ^{13}C isotope enrichment ($\delta^{13}\text{C}$, ‰) of samples was calculated as:

$\delta^{13}\text{C} = ((R_{\text{sample}} - R_{\text{standard}}) / (R_{\text{standard}})) \times 1000$, where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample, and R_{standard} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the reference standard calibrated against the international standard V-PDB (Pee Dee Belemnite). The ^{13}C atom percent excess (APE, ‰) of the samples was determined as the difference between the atom percentage (^{13}C atm‰) of the enriched sample and the un-enriched sample ('blank'), according to:

$$\text{APE } (^{13}\text{C}) = ^{13}\text{C atm‰}_{\text{sample}} - ^{13}\text{C atm‰}_{\text{blank}}$$

Atomic percentages were calculated as:

$^{13}\text{C atm‰} = (100 \times \text{AR} \times ((\delta^{13}\text{C} / 1000) + 1)) / (1 + \text{AR} \times ((\delta^{13}\text{C} / 1000) + 1))$, where AR is the absolute $^{13}\text{C}/^{12}\text{C}$ ratio of V-PDB (0.0112372) as given by Craig⁽⁴⁰⁾.

Lipid deposition of protein origin expressed as a fraction of total lipid deposited (LDPO, %) was calculated according to:

$\text{LDPO} = ((\text{BM}_{\text{end}} \times \text{BL}_{\text{end}} \times \text{C}_{\text{wbl}} \times ^{13}\text{C APE}_{\text{wbl}}) / \text{PE}_{\text{diet}}) / (\text{FI}_{\text{enr}} \times \text{DL} \times \text{ADC}_{\text{lipid}} \times \text{aDLR} \times \text{C}_{\text{wbl}})$,
where: BM_{end} = the end biomass (in g); BL_{end} = the end body lipid content (in %); C_{wbl} = the end

whole body lipid carbon content (in %); $^{13}\text{C APE}_{\text{wbl}}$ = the ^{13}C atom percent excess in the whole body lipid fraction of the fish (in %) at the end of the 3 day enrichment period; PE_{diet} = the ^{13}C enrichment of dietary protein (in %), FI_{enr} = the intake of ^{13}C enriched feed (in g), DL = the dietary lipid content (in %); $\text{ADC}_{\text{lipid}}$ = the apparent digestibility coefficient of dietary lipid (in %); and aDLR = the apparent digestible lipid retention (in %) obtained from the growth trial.

The recovery of carbon derived from dietary protein in whole fish lipid (RPCL, %) was calculated according to:

$$\text{RPCL} = (\text{BM}_{\text{end}} \times \text{BL}_{\text{end}} \times \text{C}_{\text{wbl}} \times ^{13}\text{C APE}_{\text{wbl}}) / (\text{FI}_{\text{enr}} \times \text{C}_{\text{diet}} \times ^{13}\text{C APE}_{\text{diet}} \times \text{ADC}_{\text{protein}})$$
, where: C_{diet} = the diet carbon content (in %); $^{13}\text{C APE}_{\text{diet}}$ = the ^{13}C atom percent excess of the enriched diets (in %); and $\text{ADC}_{\text{protein}}$ = the apparent digestibility coefficient of protein (in %).

The apparent nutrient digestibility coefficient of nutrient X ($\text{ADC}_{(\text{X})}$) was calculated according to:

$$\text{ADC}_{(\text{X})} = 1 - ((\text{I}_{\text{diet}} \times \text{X}_{\text{faeces}}) / (\text{I}_{\text{faeces}} \times \text{X}_{\text{diet}}))$$
, where I_{diet} and I_{faeces} is the yttrium concentration recovered in the diet and faeces, respectively, and X_{faeces} and X_{diet} is the concentration of X (protein, lipid, starch or carbon isotope) recovered in the faeces and diet, respectively⁽⁴¹⁾.

3.6 Statistical analysis

Data on FCR, SGR, ADC, DPR, aDLR, DER, digestible energy intake (DEI), lipid deposition of protein origin (LDPO), recovery of carbon derived from dietary protein in whole fish lipid fraction (RPCL), and proximate composition of whole fish were subjected to two-way ANOVA analysis to test for main effects of, and interactions between, dietary DE and DP, respectively. Significant differences caused by a main effect were subsequently assessed using the Holm-Sidak all pairwise multiple comparisons test. A probability of $P < 0.05$ was considered significant in all analyses.

4. Results

4.1 Diets and dietary ^{13}C enrichment

The ingredient composition and chemical and isotope analyses of the experimental diets are shown in table 1. The nine experimental diets were designed to comprise three different DP levels combined with three different DE levels in a 3×3 factorial design. The crude protein content of the LP, MP and HP diets ranged between 372 – 385, 404 – 409 and 443 – 453 g/kg feed, respectively. Similarly, the DE levels ranged between 19.6 – 19.9, 20.6 – 21.2 and 21.3 – 21.6 MJ/kg feed for LE, ME and HE diets, respectively. Collectively, the nine experimental diets covered a DP/DE

range from 15.6 to 20.6 g/MJ. The dietary carbon content ranged between 46.2 and 51.3%. The $\delta^{13}\text{C}$ values of the non-enriched diets ranged between -22.5 and -21.3‰, while $\delta^{13}\text{C}$ values of the enriched diets ranged between 593.9 and 754.5‰, corresponding to ^{13}C APE values from 0.673 to 0.846%. The measured ^{13}C enrichment of dietary protein carbon ranged between 1.718 to 2.010%. The indispensable amino acid (IAA) profile of the nine experimental diets are presented in figure 2. Also, the IAA profile of the *Spirulina* protein isolate employed and the IAA requirements of gilthead sea bream approximated by Kaushik⁽³⁾ is presented.

4.2 Digestibility of macro nutrients, energy and carbon isotopes

The ADCs of macro nutrients and stable carbon isotopes are shown in table 2. Protein, lipid and starch ADCs ranged from 88.7 – 89.8%, 94.1 – 95.3% and 94.6 – 95.7%, respectively, and neither were significantly affected by dietary treatment. ADCs of the two stable carbon isotopes, ^{12}C and ^{13}C , were 84.8 and 84.3%, respectively. No significant differences between the two carbon isotope ADCs were observed.

4.3 Feeding, growth, feed conversion ratio and mortality

Results on SGR, FCR and total DEI from the 89 days feeding trial are presented in table 2. FCRs (ranging from 1.24 – 1.48) were significantly lowered by increasing DE in all digestible protein groups (LP, MP and HP), while no significant effects of DP was observed in FCRs. SGRs (ranging from 0.96 – 1.05%/d) were slightly, but significantly, higher with increasing DP in fish fed LE diets, while no significant effects of DE on SGRs were observed for any of the dietary treatment groups. DEI ranged between 82.5 and 87.7 MJ, and was significantly different among DP groups. No mortality occurred throughout the trial.

4.4 Chemical composition of fish

The chemical composition of whole fish at the beginning and at the end of the 89 days growth period are presented in table 3. A two-way ANOVA analysis showed no significant effects of dietary treatment on whole body lipid, ash or DM, while the whole body protein content was significantly higher in fish fed HP diets than in fish fed LP and MP diets. After 3 days of feeding using diets with a ^{13}C enriched protein content, ^{13}C APE in lipid fraction of whole fish ranged between 8.69 and $13.04 \times 10^{-3}\%$ (table 3).

280 4.5 Nutrient retention efficiencies

281 Results on DPR and aDLR based on the 89 day feeding trial, are presented in table 4 and in figure
282 1: i and ii. DPRs ranged between 30.6 and 39.2%, and was significantly affected both by dietary DP
283 and DE level, showing increased retention efficiency with increasing DE and decreasing DP (figure
284 1: i). aDLR ranged between 70.4 and 95.1%, and was also significantly affected both by dietary DP
285 and DE level, showing increased retention efficiency with increasing DP within the LE groups
286 and/or with decreasing DE within the MP and HP groups (figure 1: ii). Results on DER are also
287 presented in table 4 ranging between 48.0 and 54.9%. DER was not significantly affected by dietary
288 treatment.

289 4.6 Recovery of protein derived carbon in fish lipid (RPCL) and contribution to total lipid 290 deposition (LDPO) of lipid synthesized from dietary protein *de novo*

291 The recovery of carbon derived from dietary protein in whole fish lipid (RPCL) as determined from
292 the stable isotope analyses ranged between 18.6 and 22.4% and was significantly affected by DE
293 and the interaction between DP and DE. Pairwise comparisons showed that RPCL was significantly
294 increased by increasing DP in LE group and by decreasing DE in MP group (table 4; figure 1: iii).
295 The contribution to total lipid deposition from lipid synthesized from dietary protein *de novo*
296 (LDPO) ranged between 21.6 and 30.2%, and was significantly affected by both dietary DP and
297 DE, showing increased contribution with decreasing DE and/or increasing DP (table 4; figure 1: iv).

298 5. Discussion

299 The main purpose of the present study was to determine: 1) the magnitude of *de novo* lipogenesis
300 originating from dietary protein in gilthead sea bream fed nine diets with different DP (330, 360 or
301 380 g/kg) and DE (20, 21 or 22 MJ/kg) levels set up in a 3×3 factorial design; and 2) to determine
302 the overall contribution to lipid deposition from *de novo* lipogenesis originating from dietary
303 protein in this species. Simple, high quality raw material matrices were applied to assure highest
304 possible quality of dietary nutrients and to avoid possible anti-nutritional effects associated with
305 certain vegetable raw materials⁽⁴²⁾. DP/DE levels of the nine experimental diets were deliberately
306 formulated to also cover a lower range (from 15.6 to 20.1 g/MJ) than previously recommended for
307 gilthead sea bream by e.g. Lupatsch *et al.*⁽¹²⁾ (ranging from 19.0 to 22.6 g/MJ for present size fish).
308 This was done in order to incite possible effects of protein deficiency on *de novo* lipogenesis,
309 performance and nutrient retention efficiencies. Also, recommendations on dietary DE densities
310 from the same authors were slightly more conservative (ranging between 15 and 20 MJ/kg), than
311 dietary DE densities of the present study (ranging between 19.6 and 21.6 MJ/kg).

312 The dietary indispensable amino acid (IAA) profile of all experimental diets satisfied the
 313 requirements put forward by Kaushik⁽³⁾. However, since these recommendations were expressed
 314 relatively to dietary nitrogen content, fish fed low DP/DE diets might have experienced a general
 315 lack of DP.

316 The present study clearly demonstrated that DP, irrespective of diet, did indeed contribute
 317 significantly to endogenous lipid biosynthesis in gilthead sea bream, as seen both from the recovery
 318 of DP-carbon in the lipid fraction of the fish (RPCL) and from the contribution of lipid synthesized
 319 *de novo* to total lipid deposition (LDPO) (table 4 and figure 1: iii and iv). The results thereby
 320 corroborate the findings by Enes *et al.*⁽³⁰⁾ and Figueiredo-Silva *et al.*⁽²⁷⁾ who both observed a
 321 significant correlation between hepatic lipogenic enzyme activity (glucose-6-phosphate
 322 dehydrogenase (G6PD)) and dietary protein level in diets for gilthead sea bream and blackspot
 323 seabream, respectively. These studies, including the present, are thereby in contrast to the review by
 324 Tocher⁽⁴³⁾, who claims that biosynthesis of fatty acids *de novo* is not likely to occur to any
 325 significant extent in marine predatory species. Also, using ¹³C labelled dietary protein, Campbell⁽²⁶⁾
 326 found that between 9.7 – 44.5% of whole body lipids in rainbow trout (*Onchorynchus mykiss*)
 327 juveniles were derived from dietary protein, using diets with a protein-to-energy ratio ranging
 328 between 17.7 and 26.6 g/MJ, respectively. In the present study approximately one-fifth (18.6 to
 329 22.4%) of the dietary DP supplied was converted into body lipid irrespective of dietary treatment.
 330 Fish fed diets MPLE and HPLE displayed slightly higher RPCL values (22.4 and 21.1%,
 331 respectively) than fish fed the remaining seven diets. This might have been due to these two diets
 332 having the highest DP/DE level in their respective DE groups, triggering excessive protein
 333 deamination and donation of extra carbon for lipid biosynthesis (table 4 and figure 1: iii). The
 334 contribution of lipid synthesized *de novo* from DP to the total lipid deposition (LDPO) ranged
 335 between 21.6 – 30.2%, confirming that *de novo* lipid synthesis from DP plays a major role in the
 336 overall lipid deposition in gilthead sea bream. The LDPO was clearly elevated in the low energy
 337 diets of each DP group, and by increasing DP generally (table 4 and figure 1: iv). Thus, LDPO
 338 values were directly related to dietary DP/DE levels. This was also reflected in the aDLR values
 339 which ranged between 70.4 to 95.1% (table 4 and figure 1: ii). Hence, similar to the LDPO results,
 340 aDLR increased with increasing dietary DP and decreasing DE (i.e. increasing DP/DE ratio).
 341 Conversely, the DPR results showed increasing retention efficiencies with decreasing DP and/or
 342 increasing DE level (i.e. decreasing DP/DE ratio). These results substantiate the so-called protein
 343 sparing effect of substituting DE originating from DP with DE from non-protein sources as already
 344 reported in a number of aquacultured species^(23, 44, 45), including gilthead sea bream^(19, 24, 46) (table 4
 345 and figure 1: i). Thus, the aDLR and DPR results combined suggest that while protein was spared
 346 by a decreasing dietary DP/DE level, the opposite was true for lipid, substantiating that deaminated

DP was indeed converted into body lipids. The result of these opposing nutrient retention dynamics have supposedly rendered differences in the overall DERs insignificant, as shown in table 4. In addition, the proximate composition of whole fish was largely unaffected by dietary treatment. No significant effects were observed in whole body lipid, ash and dry matter, while a very small, but significant, effect of dietary DP was observed on whole body protein content. This is in accordance with the finding of Bonaldo *et al.*⁽⁴⁷⁾, who found no difference in body composition of gilthead sea bream fed 3 diets differing in dietary DP/DE ratio for 81 days. Thus, considering lipogenesis, nutrient retention and body composition results of the present study collectively, fish appeared to endeavour to rigorously maintain a certain whole body energy status under a wide variety of dietary DP/DE ratios, even if substantial amounts of dietary protein was sacrificed to achieve this.

It was expected that the SGR values obtained from the growth period would not differ significantly among dietary treatment groups, as fish were fed iso-DE throughout the trial. However, a small, but significant, difference was observed between the LPLE and HPLE fish. This could be partly explained by the slightly lower DEI observed in fish fed the LPLE diet (83.5 MJ) compared to fish fed the HPLE group (87.0 MJ), or by a possible general lack of DP experienced by LPLE fish. However, since dietary DP level did not have any significant effect on the FCRs obtained from the growth trial, the latter point probably does not apply. In contrast, FCRs were clearly improved by a dietary DE increase. This clear link between dietary DE and FCR response have been reported earlier in a number of aquacultured fish species^(17, 48), including gilthead sea bream^(12, 13) when growth was not limited by dietary protein content. The present results thereby indicate that gilthead sea bream have the ability to efficiently utilize diets with lower DP/DE ratios and higher energy densities (virtually resembling commercial diets for salmonid species) than previously recommended⁽¹²⁾ without showing adverse effects on proximate composition or performance of the fish.

The measured ADCs of protein, lipid and starch did not differ significantly among dietary treatments, which was also expected from the diet optimization, considering that the same raw materials were used in all diets, and only inclusion levels differed.

An inherent problem by using a tracer to investigate metabolic pathways is the potential difference in functional behaviour between the tracer and the tracee. In the present study uniformly ¹³C labelled *Spirulina* protein isolate was used to trace the fate of dietary protein, which mainly originated from con-kix fish meal. However, possible differences in overall protein digestibility, amino acid profile and individual amino acid digestibility between *Spirulina* protein isolate and dietary protein could potentially lead to differences in the way that tracer and tracee was metabolized, rendering the tracer unsuitable for the purpose. However, when comparing the IAA profile of *Spirulina* protein isolate and experimental diets only small differences were apparent

382 (figure 2). The only clear difference was a considerable higher lysine content of the experimental
383 diets. This difference, however, was unlikely to change the overall pattern in amino acid
384 deamination since the remaining amino acids were basically in balance, and all experimental diets
385 as such fulfilled the general IAA requirements of gilthead sea bream^(3, 4). Individual amino acid
386 ADCs were not determined in the present trial. However, the measured stable carbon isotope ADCs
387 displayed no significant differences between ¹²C and ¹³C, indicating that *Spirulina* whole protein
388 was indeed digested similarly to the remaining dietary protein fraction. Thus, it was assumed that
389 the *Spirulina* protein isolate could be considered a true tracer, not behaving functionally different
390 from the tracee.

391 **6. Conclusion**

392 For the first time orally administered ¹³C labelled protein has been applied to quantify *de novo*
393 lipogenesis originating from dietary protein and to determine the importance of this in the overall
394 body lipid deposition in gilthead sea bream. Irrespective of dietary treatment the fish converted
395 substantial amounts of carbon derived from dietary protein into body lipids, which in turn
396 contributed significantly to total body lipid deposition. Despite dietary effects on protein and lipid
397 retention efficiencies and *de novo* lipogenesis the fish were able to maintain a constant retention of
398 DE with no significant effects seen on whole body composition. The results indicate that this
399 species may have evolved to maximize energy storage (in the form of lipid) for seasonal, migratory
400 or maturation purposes at the expense of increasing body size through more efficient use of protein
401 for growth. Additionally, the improvement of FCR by increased DE combined with an
402 improvement of DPR with decreasing DP/DE level suggest that gilthead sea bream is able to
403 efficiently utilize feeds within a wide range of dietary DP/DE ratios, which could be taken into
404 consideration in future production of commercial feeds for this species.

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412 wrote the manuscript; all authors helped to proofread the manuscript. There are no conflicts of
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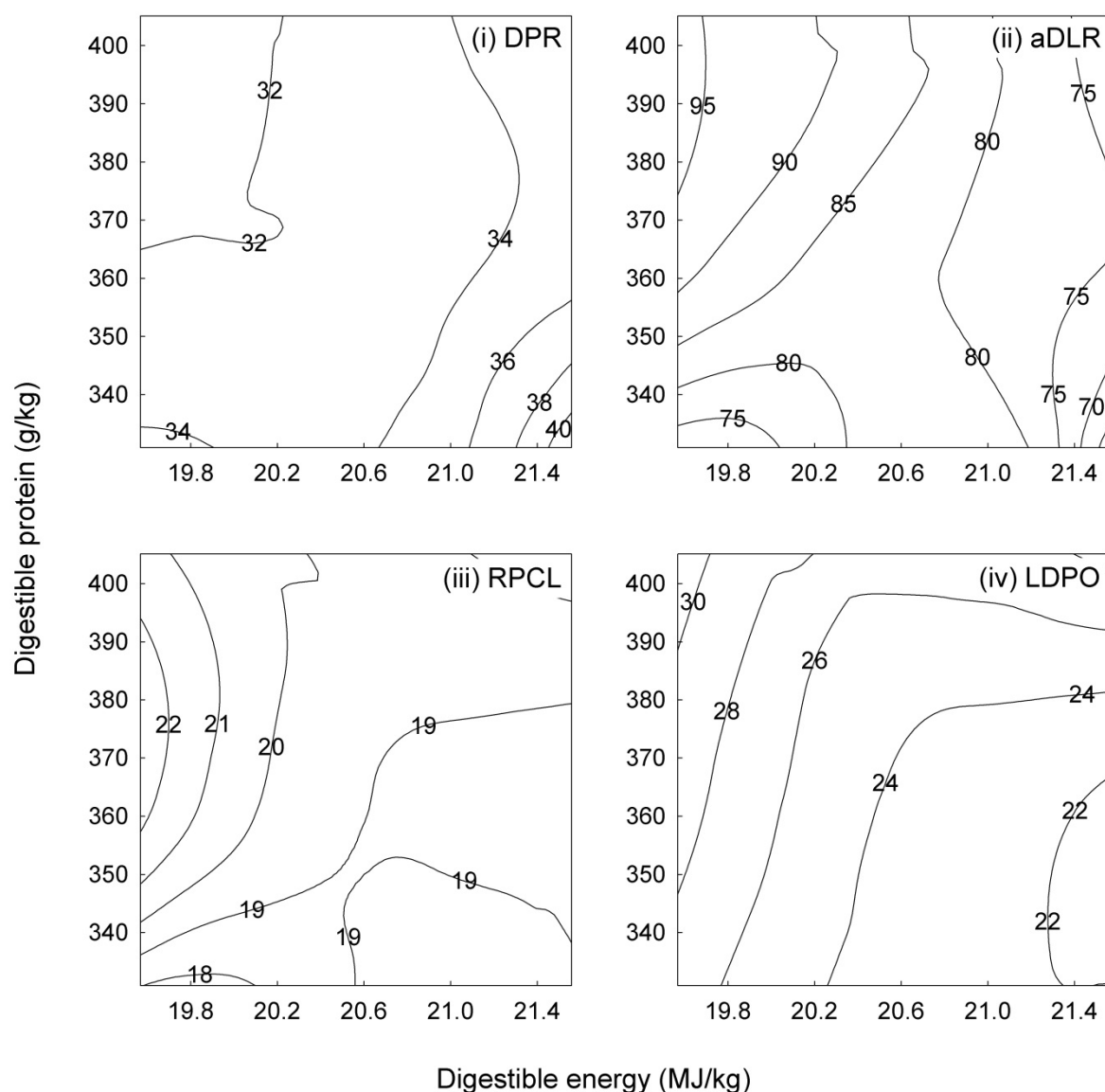
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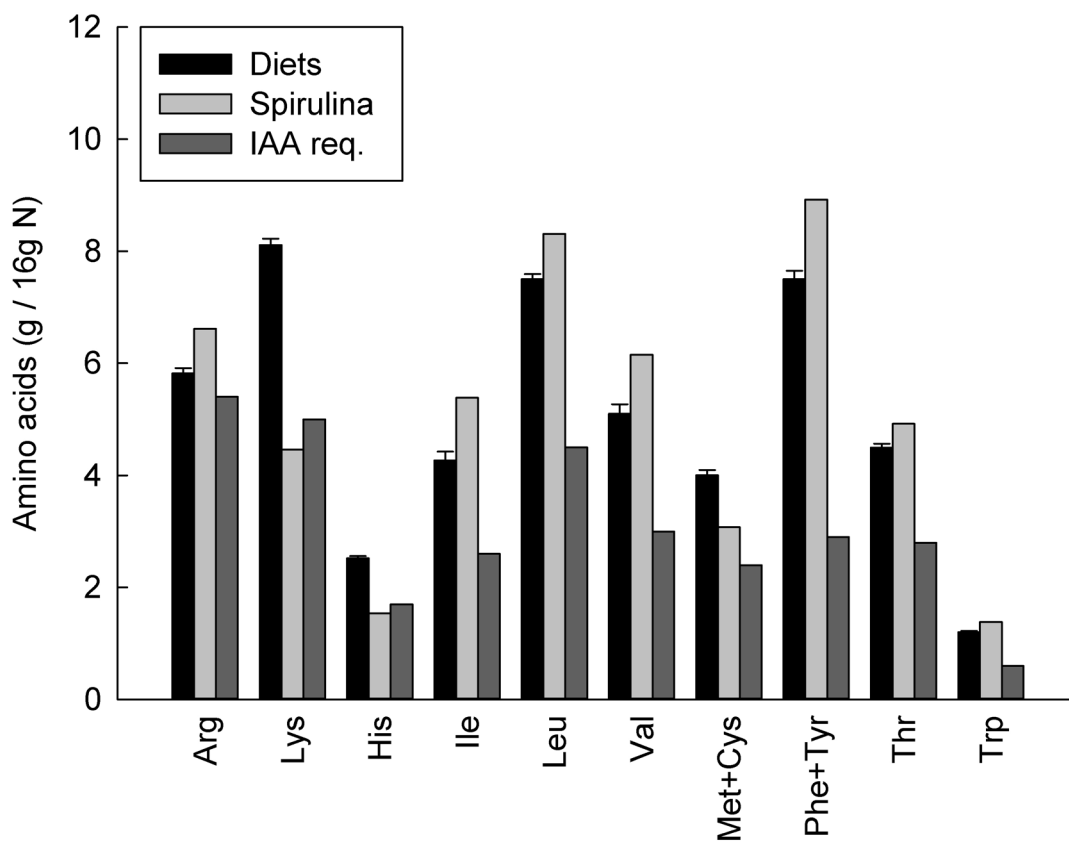
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 541 **Figure 1.** Contour plots of the effects on (i) digestible protein retention (DPR, %), (ii) apparent
 542 digestible lipid retention (aDLR, %), (iii) recovery of protein carbon in lipid fraction of fish (RPCL,
 543 %), and (iv) the percentage of total lipid deposit originating from dietary protein (LDPO, %) in fish
 544 fed 9 diets differing in digestible protein (DP) content and digestible energy (DE) content for a
 545 period of 89 feeding days. All diets were fed to triplicate tanks.



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547 **Figure 2.** The amino acid profile of diets (mean \pm STD, n=9) (black bars) and *Spirulina* protein
 548 isolate (light grey bars). The IAA requirements of gilthead sea bream as approximated by Kaushik⁽³⁾
 549 are shown as dark grey bars.

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552 **Table 1** Diet formulation, chemical and isotope composition of experimental diets

Diet	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE
Ingredients (g/kg)*									
Fish meal	442	448	453	498	504	509	554	559	565
Fish oil	180	218	257	163	202	240	146	185	223
Wheat	207	161	115	154	108	61	101	55	8
Field peas	147	149	151	166	168	170	185	186	188
Guar gum	5	5	5	5	5	5	5	5	5
Vitamin and mineral premix	3	3	3	3	3	3	3	3	3
Monocalcium phosphate	16	16	16	11	11	11	7	7	7
Y ₂ O ₃	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
¹³ C protein isolate†	(7)	(7)	(7)	(7.5)	(7.5)	(7.5)	(8)	(8)	(8)

Proximate composition (g/kg)

Dry matter	930	921	920	915	909	922	917	918	920
Crude protein	385	372	375	409	404	404	453	443	444
Crude fat	230	274	308	219	256	304	210	254	283
Starch	193	192	126	168	152	102	133	106	84
Ash	74	72	72	73	74	73	77	76	77
Energy calculations									
Gross energy (GE, MJ/kg)‡	22.4	23.1	23.9	22.0	22.7	24.0	22.1	23.0	23.7
Digestible energy (DE, MJ/kg)§	19.9	21.2	21.4	19.6	20.7	21.6	19.7	20.6	21.3
Digestible protein (DP, g/kg)	344	331	334	363	363	361	405	395	395
DP/DE ratio (g/MJ)	17.3	15.6	15.6	18.5	17.5	16.7	20.6	19.1	18.5
Carbon content of diets (%)									
Non-enriched diets	47.74	49.89	51.25	48.69	48.44	50.04	47.17	49.19	50.11
SD	1.06	1.15	1.44	0.51	0.51	1.39	0.38	1.07	0.49
¹³ C enriched diets	46.19	50.20	51.08	46.50	50.38	50.74	48.70	49.50	49.61
SD	1.01	1.29	1.36	0.63	1.20	1.64	0.74	1.12	0.82

$\delta^{13}\text{C}$ values of diets (‰)

Non-enriched diets	-22.48	-22.40	-22.23	-22.12	-21.44	-21.52	-21.25	-21.59	-21.81
SD	0.17	0.06	0.09	0.13	0.27	0.71	0.25	0.30	0.12

^{13}C enriched diets	594.54	596.93	593.91	661.68	663.74	663.23	753.28	754.48	753.25
SD	17.12	15.01	16.27	18.90	17.30	26.67	25.18	8.53	34.83

^{13}C APE of diets (%)

^{13}C enriched diets	0.6738	0.6762	0.6728	0.7461	0.7476	0.7471	0.8443	0.8459	0.8448
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^{13}C enrichment of protein C (%)

^{13}C enriched diets	1.718	1.943	1.950	1.805	1.982	1.998	1.931	2.010	2.010
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*Fish meal: TripleNine Fish Protein, Esbjerg, Denmark; Fish oil: South American (Peru); Guar gum: HV200, LCH A/S, Frederiksberg, Denmark; ^{13}C labelled protein isolate: Cambridge Isotope Laboratories Inc., Algal crude protein fraction isolated from *Spirulina* algae (U- ^{13}C , 97-98%), lot no. BP-733, cat. no. CLM-3348-0, Cambridge Isotope Laboratories, Inc., Andover, MA, USA; Vitamin and mineral mix is estimated to meet the requirements according to the U.S. National Research Council recommendations⁽⁴⁹⁾.

†Each of the nine diets were produced in a ^{13}C enriched version (adding ^{13}C labeled protein isolate) and a non-enriched version (adding con-kix fish meal) in order to determine ^{13}C APE of experimental diets.

‡Gross energy (GE, MJ/kg) was calculated as the sum of the dietary content of protein, lipid and nitrogen-free extract (NFE), multiplied by their respective energetic values upon complete oxidation⁽⁵⁰⁾. $\text{GE} = (\text{P}_{\text{diet}} \times 23.66) + (\text{L}_{\text{diet}} \times 39.57) + (\text{NFE}_{\text{diet}} \times 17.17)$, where P_{diet} , L_{diet} and NFE_{diet} refer to

the dietary protein, lipid and NFE content (%), respectively. NFE was calculated as the sum of dietary protein, lipid, ash and water deducted from 100% (by difference).

§The digestible energy (DE, MJ/kg) content was calculated as the dietary gross energy, but with the ADC of each nutrient multiplied into their respective terms:

$$DE = (P_{\text{diet}} \times 23.66 \times \text{ADC}_{\text{protein}}) + (L_{\text{diet}} \times 39.57 \times \text{ADC}_{\text{lipid}}) + (S_{\text{diet}} \times 17.17 \times \text{ADC}_{\text{starch}}), \text{ where } S_{\text{diet}} \text{ is the dietary starch content, and } \text{ADC}_{\text{protein}},$$

$\text{ADC}_{\text{lipid}}$ and $\text{ADC}_{\text{starch}}$ are the ADCs of protein, lipid and starch, respectively.

$$||\text{Digestible protein (DP, g/kg)} = \text{dietary crude protein content} \times \text{ADC}_{\text{protein}}$$

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Table 2 Feed conversion ratio (FCR), specific growth rate (SGR), digestible energy intake (DEI), and apparent nutrient digestibility coefficients (ADCs) of macro nutrients and stable carbon isotopes (mean \pm STD, n=3)

Diets	2-way ANOVA*										
	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	DP	DE
Specific growth rate (%/d) and digestible energy intake (MJ) during growth period											
FCR†	1.48 ^x	1.32 ^y	1.27 ^y	1.42 ^x	1.38 ^{xy}	1.29 ^y	1.38 ^x	1.29 ^{xy}	1.24 ^y	0.052	<0.001
SD	0.03	0.06	0.05	0.03	0.02	0.09	0.07	0.03	0.05		
SGR‡	0.96 ^a	1.02	1.02	1.01 ^{ab}	0.99	1.01	1.03 ^b	1.05	1.04	0.028	0.336
SD	0.01	0.04	0.04	0.01	0.01	0.06	0.04	0.01	0.03		0.266
DEI§	83.5	86.8	82.5	85.1	83.7	85.7	87.0	87.7	87.0	0.027	0.584
SD	2.9	1.4	1.7	1.5	2.6	2.1	2.2	2.3	2.8		0.218
Apparent digestibility coefficients of dietary macro nutrients and stable carbon isotopes (%)											
Protein	89.2	89.0	89.1	88.7	89.8	89.3	89.4	89.0	89.1	0.957	0.959
SD	2.2	0.8	0.8	0.9	1.6	1.1	0.7	0.5	1.0		0.849
Lipid	95.0	94.6	94.1	95.0	95.1	94.5	95.3	94.9	94.9	0.605	0.482
SD	1.6	0.5	1.1	0.6	0.4	1.1	1.8	0.7	0.7		0.969
Starch	94.8	95.7	94.8	95.7	95.5	94.7	95.2	95.5	94.6	0.933	0.307
SD	1.4	1.0	0.2	0.8	0.7	0.9	1.3	1.9	1.5		0.924

Table 3 Chemical and isotope composition of whole fish (mean ± STD, n=3) *

Diets	2-way ANOVA *										
	Initial	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	DP×DE
Proximate composition of fish (%)											
Protein	17.0	17.1	16.9	16.9	17.2	17.1	17.1	17.3	17.2	17.4	0.826
SD		0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.2	
Lipid	16.4	21.5	22.3	22.3	21.9	21.5	21.9	21.9	21.4	20.6	0.657
SD		0.9	0.2	0.8	1.3	0.6	0.7	0.8	0.8	1.6	
Ash	3.4	3.4	3.3	3.2	3.3	3.3	3.3	3.3	3.3	3.2	0.749
SD		0.1	0.2	0.1	0.1	0.1	0.1	0.0	0.1	0.1	
Dry matter	35.6	40.8	41.5	41.4	41.0	41.0	41.1	41.2	41.1	40.0	0.487
SD		0.4	0.7	0.5	0.8	0.5	0.8	0.7	0.6	1.0	
Carbon content of whole fish lipid fraction (%)											
Pre-enrichment		68.30	67.42	67.43	68.03	68.11	68.08	69.08	68.50	68.33	
SD		0.75	0.44	0.25	0.93	1.63	0.89	0.83	1.08	1.44	
Post-enrichment		70.01	69.86	69.40	69.57	69.32	68.55	69.84	68.70	68.32	
SD		0.14	0.29	0.21	0.11	0.06	0.34	1.04	0.61	0.25	

$\delta^{13}\text{C}$ values of whole fish lipid fraction (‰)

Pre-enrichment	-23.75	-23.50	-23.33	-23.80	-23.57	-23.34	-23.75	-23.58	-23.36
SD	0.03	0.06	0.06	0.01	0.06	0.02	0.05	0.05	0.04
Post-enrichment	-15.84	-15.11	-15.05	-13.17	-13.87	-14.47	-11.88	-12.53	-12.04
SD	0.13	0.46	0.60	0.43	0.78	0.31	0.38	0.47	0.51

^{13}C atom percent excess (APE) in whole fish lipid fraction ($\times 10^{-3}\%$)

Post-enrichment	8.69	9.23	9.10	11.68	10.67	9.75	13.04	12.15	12.44
SD	0.11	0.58	0.60	0.48	0.92	0.32	0.39	0.46	0.57

*Two-way ANOVA analysis (df = 2,26) on effects of digestible protein (DP), digestible energy (DE) and their interaction (DP \times DE). Significant effects of DE within DP groups were denoted superscripted x and y, while significant effects of DP within DE groups were denoted superscripted a and b (Holm-Sidak method, $P < 0.05$).

Table 4 Digestible macro nutrient retention and recovery of protein derived carbon in whole fish lipid fraction (mean \pm STD, n=3)^{*}

Diets	2-way ANOVA*											
	LPLE	LPME	LPHE	MPLE	MPME	MPHE	HPLE	HPME	HPHE	DP	DE	DP×DE
Retention of digestible protein (DPR, %) [†]												
DPR	33.2 ^x	37.3 ^{ay}	39.2 ^{ay}	32.1 ^x	32.6 ^{bx}	35.4 ^{by}	30.6 ^x	33.0 ^{bx}	34.5 ^{by}	<0.001	<0.001	0.237
SD	0.8	1.5	1.0	1.0	0.7	2.5	2.3	0.3	0.5			
Apparent retention of digestible lipid (aDLR, %) [†]												
aDLR	79.4 ^a	78.4	70.4	92.2 ^{bx}	80.5 ^y	74.7 ^y	95.1 ^{bx}	86.3 ^{xy}	76.0 ^y	0.007	<0.001	0.448
SD	8.7	1.7	4.5	6.0	2.2	2.1	6.4	7.2	7.9			
Digestible energy retention (DER, %) [†]												
DER	48.0	51.6	52.1	52.8	51.0	53.4	53.2	54.9	53.0	0.101	0.524	0.405
SD	3.8	1.2	2.7	3.0	1.3	1.9	3.3	3.4	3.9			
Recovery of carbon originating from dietary protein in lipid fraction of fish												
RPCL (%)	19.2 ^a	19.8	19.3	22.4 ^{bx}	18.9 ^y	18.6 ^y	21.1 ^b	19.5	19.8	0.243	0.001	0.005
SD	0.9	0.7	0.3	0.9	0.9	0.2	1.0	0.8	1.5			
LDPO (%)	25.9 ^{ax}	22.1 ^{ay}	21.9 ^{ay}	28.8 ^{bx}	23.4 ^{aby}	21.6 ^{az}	30.3 ^{bx}	25.2 ^{by}	26.3 ^{by}	0.002	<0.001	0.542
SD	2.4	1.2	0.9	2.3	1.9	1.1	2.5	2.2	2.5			

602 *Two-way ANOVA analysis (df = 2,26) on effects of digestible protein (DP), digestible energy (DE) and their interaction (DP×DE). Significant effects
603 of DE within DP groups were denoted superscripted x and y, while significant effects of DP within DE groups were denoted superscripted a and b
604 (Holm-Sidak method, $P < 0.05$).

605 †The digestible protein retention (DPR), apparent digestible lipid retention (aDLR) and digestible energy retention (DER) were calculated as the ratio
606 between the amount of protein, lipid and energy retained by the fish and the amount of protein, lipid and energy digested by the fish during the growth
607 trial, respectively.

4. Paper III: Effects of substituting lipid with starch as dietary energy source on performance, nutrient utilization and fatty acid composition in gilthead sea bream (*Sparus aurata*) juveniles

Effects of substituting dietary lipid with starch on performance, nutrient utilization and fatty acid composition in gilthead sea bream (*Sparus aurata*) juveniles

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1. Abstract

This study examined the effects of a gradual substitution of dietary lipid with carbohydrate on feed and growth performance, nutrient utilization and fatty acid composition in juvenile gilthead sea bream (*Sparus aurata*). Triplicate groups of fish were fed four extruded iso-energetic diets (2.7%/d) in which lipid was gradually reduced (from 228 to 151 g kg⁻¹) and replaced by increasing amounts of starch (from 52 to 230 g kg⁻¹). The experiment lasted 41 days, and fish grew from 25 to 75 grams at a temperature of 24°C. The feed conversion ratio (ranging from 1.00 – 1.03), viscerosomatic index (ranging from 7.85 – 8.56%), and proximate composition of whole fish were not affected by the dietary treatment ($p > 0.05$), while specific growth rate (ranging from 2.61 – 2.66% d⁻¹) was slightly reduced with increasing dietary starch intake ($p = 0.041$). Apparent retention of saturated fatty acids (SAFAs) and mono-unsaturated fatty acids (MUFAs) exceeded 100% in fish fed high starch diets, indicating substantial *de novo* lipogenesis and $\Delta 9$ desaturase activity. In comparison, apparent retention of poly-unsaturated fatty acids (PUFAs) was unaffected by the dietary treatment (ranging between 57.0 – 62.4%). As a result, fish growing from 25 to 75 g displayed increasing SAFA and MUFA levels and decreasing PUFA levels with increased dietary starch inclusion. Applied to marked size fish, such changes could compromise flesh quality and thus market value of cultured gilthead sea bream.

2. Introduction

Aquaculture is the fastest growing food industry in the world (FAO, 2010), but exerts a huge demand for limited resources of fish meal and fish oil as feed ingredients, which have caused prices to triple within the last decade. A substantial number of studies have been undertaken to find suitable fish meal and fish oil replacements, including protein from vegetable raw materials (Gatlin III. et al., 2007), animal by-products (Bureau et al., 1999; El-Haroun et al., 2009), single-cell organisms (Kiessling and Askbrandt., 1992; Perera et al., 1995; Aas et al., 2006), and lipids of plant origin (Corraze and Kaushik, 2009; Turchini et al., 2009). Macronutrients in the natural diet of marine carnivorous fish comprise mainly protein and lipids abundant in essential amino acids (EAA) and essential fatty acids (EFA), respectively, while they are basically free of anti-nutrients. The introduction of vegetable raw materials into aquaculture feeds has caused a concomitant increase in dietary carbohydrate, challenging the carnivorous nature of the digestive and oxidative processes associated with metabolism in many cultured species (Hemre et al., 2002; Stone, 2003). However, several studies on gilthead sea bream (*Sparus aurata*) have demonstrated excellent starch digestibility coefficients (Venou et al., 2003; Enes et al., 2008a; Adamidou et al., 2009), as well as presence and activity of the major enzymes of the glycolytic pathway (Panserat et al., 2000; Couto et al., 2008; Enes et al., 2008a; Enes et al., 2008b; Enes et al., 2008c). These findings indicate a possible efficient utilization of dietary starch for energy purposes. Additionally, this species appears to be able to utilize dietary carbohydrates for endogenous lipid biosynthesis as indicated by an enzymatic up-regulation of hepatic glucose-6-phosphate dehydrogenase (G6PD) with increasing dietary carbohydrate levels (Meton et al., 1999; Fernandez et al., 2007; Enes et al., 2008a; Enes et al., 2008b; Enes et al., 2011). This was corroborated in a recent study using ¹³C labelled starch as a

metabolic tracer (Ekman et al., 2012). The study showed that between 5-8% of the dietary starch carbon was recovered in the whole body lipid fraction of juvenile gilthead sea bream using diets similar to those of the present study. Furthermore, results from the study strongly indicated that dietary protein may be another potent carbon donor in *de novo* lipogenesis in gilthead sea bream.

Irrespective of dietary carbohydrate or protein being the original carbon source (i.e., *via* oxidative decarboxylation of pyruvate or oxidative degradation of certain amino acids, respectively), acetyl-CoA is the ultimate carbon donor for biosynthesis of lipids *de novo*. The main products of such *de novo* lipogenesis are 16:0 (palmitic acid) and 18:0 (stearic acid) (Sargent et al., 1989), which subsequently may be desaturated to 16:1(n-7) and 18:1(n-9), and possibly undergo chain elongation yielding 18:1(n-7), 20:1(n-9), 22:1(n-9) and 24:1(n-9) (Tocher, 2003). The extent to which FAs are biosynthesized endogenously, and subsequently desaturated and chain elongated, could potentially inflict an adverse effect on the FA profile of cultured fish compared to fish caught in the wild. This could compromise the marked value of cultured gilthead sea bream, considering the increasing awareness of the beneficial / human health promoting effects of particularly (n-3) PUFA in such products.

The main purpose of the present study was to assess the effects of substituting dietary energy provided from lipid (fish oil) with dietary energy provided from carbohydrate (wheat starch) on the FA profile and apparent FA retention efficiencies in juvenile gilthead sea bream. To achieve this, fish were fed four iso-energetic diets for 41 days (corresponding to a tripling of biomass) differing by an increasing content of starch and decreasing content of lipid. Additionally, crude macro nutrient retention efficiencies, growth and feed performance, and proximate composition of fish were determined.

As deposited lipid/FAs could originate from both dietary and endogenous sources, all lipid/FA retention efficiencies are henceforth referred to as "apparent". Furthermore, apparent FA retention values exceeding 100% were perceived mainly as indicators of endogenous fatty acid biosynthesis, neglecting effects of possible FA chain shortening.

3. Materials and methods

3.1 Culture conditions and fish

Gilthead sea bream juveniles with an average individual weight of 15 g were obtained from a commercial fish farm (Ferme Marine de Douhet, Île d'Oléron, France) and transferred to the BioMar research facility at the North Sea Research Centre, Hirtshals, Denmark. The fish were stocked at a density of 85 fish tank⁻¹ in a recirculation aquaculture system comprising 12 fibre glass tanks with a volume of 800 L each, and a system water exchange rate of 3% d⁻¹. The tanks were designed to quickly and efficiently remove faeces and un-eaten feed pellets from the water *via* a central bottom drain. Externally mounted swirl separators made it possible to collect and quantify un-eaten feed pellets. The trial facility was supplied with filtered North Sea water with a salinity of 34 g L⁻¹, and temperature was kept at 24°C throughout the experiment. Water quality was monitored daily, maintaining O₂ saturation between 80-100%, ammonium below 1.0 mg L⁻¹, nitrite below 1.0 mg L⁻¹, and nitrate below 100 mg L⁻¹. pH was adjusted to 7.0 using sodium bicarbonate when necessary. Tanks were supplied with system water at a flow rate of 1200 L tank⁻¹ h⁻¹. A 14 h light : 10 h dark photoperiod was maintained throughout the study. All fish were acclimated to the facility for 2 weeks, during which they were fed a commercial diet (BioMar EFICO Sigma 860) at 3% of the estimated biomass d⁻¹.

3.2 *Experimental diets*

Four experimental diets were prepared using the Allix² feed formulation software from A-systems S.A., France (Table 1). The four diets named A, B, C and D were formulated to contain 60, 120, 180, and 240 g starch kg⁻¹ feed, respectively. Fish oil and cellulose were used to balance dietary energy levels keeping total dietary energy originating from protein, lipid and starch similar in all diets. The diets were supplemented with a vitamin and mineral premix as well as mono calcium phosphate. Guar gum was added (5 g kg⁻¹) to enhance pellet stability when assessing feed waste. Diets were prepared at the BioMar TechCenter (Brande, Denmark) using a twin screw extruder (Cleextral BC-45, Firminy, France) to produce 3 mm pellets. Following extrusion, diets were dried in a six-level Geelen counter flow continuous dryer (Geelen Counterflow, Haelen, The Netherlands), vacuum coated with fish oil, and cooled.

3.3 *Experimental procedures*

Five randomly chosen fish were removed from each of the 12 tanks at start of the experiment, euthanized using 250 mg L⁻¹ tricaine methanesulfonate (MS-222), weighed, and immediately frozen in liquid nitrogen. The 5×12 fish were subsequently pooled and stored at -20°C until analysis, constituting the initial fish sample. The remaining fish in each tank were weighed, and each of the four experimental diets was fed restrictively to triplicate tanks for 41 days at a ration adjusted according to the expected daily biomass gain in each tank (approximately 2.7% d⁻¹). Fish were fed from 08:00 to 14:00 h using automatic belt feeders. Any un-eaten feed was collected daily and subtracted in calculations of feed intake (FI) and feed conversion ratio (FCR). At the end of the study, fish were starved for 48 h and bulk weighed. Seven fish from each tank were removed, euthanized (MS-222; 250 mg L⁻¹), immediately frozen in liquid nitrogen and subsequently stored at -20°C for further fractionation and chemical analysis. Mortality

was 0.42% of the total number of individuals throughout the experiment and was not related to dietary treatment groups. All experimental procedures were carried out in accordance to EC directive 86/609/EEC for animal experiments (European Commission, 1986).

3.4 Sample preparation and chemical analysis

Feed samples were homogenized prior to analysis using a Krups Speedy Pro homogenizer. Crude protein was determined according to ISO (2005), crude fat according to Bligh & Dyer (1959), and dry matter and ash according to Kolar (1992). Phosphorus was carried out according to ISO (1998) and DS (2002), crude fibre according to EC (2009), and starch analyses according to the method of Bach Knudsen (1997). Fatty acid analysis of feed was carried out according to ISO (2002) (modified) using GC MS to obtain the full fatty acid content and amount of each FA.

Fish sampled by the end of the 41-days study were partly thawed and fractionated into livers, viscera (excluding liver), and carcass, and all fractions were weighed to determine hepatosomatic (HSI) and viscerosomatic (VSI) indices. Tissues were kept at or below 0°C during fractionation in order to avoid enzymatic degradation of glycogen. The 3 fractions were pooled at tank level (hereafter referred to as “whole fish”) subsequently to weighing, and immediately re-frozen at -20°C prior to further analysis. Frozen, whole fish samples were homogenized for 60 seconds using a Tecator 1094 homogenizer (Tecator AB, Höganäs, Sweden), and an aliquot of each sample was further homogenized for 30 seconds using a Büchi Mixer B-400 (BÜCHI Labortechnik AG, Switzerland). The aliquots were analysed as described below. Utmost care was taken during homogenization to assure that all samples stayed below 0°C to avoid enzymatic degradation of glycogen in the homogenate.

Fish sampled at the start of the 41-days study were not fractionated, but homogenized directly as described in the two-step homogenization procedure above. Crude protein, crude fat, dry matter and ash were determined as described for feed samples, while glycogen analysis were determined using a BioVision glycogen assay kit (cat. #K646-100 / Tecan GENios micro plate reader (Austria) fitted with a 570 nm colorimetric filter). Fatty acid analysis of fish sampled at start and end of the study was carried out according to ISO (2002) as described for the feed samples.

3.5 Calculations

Crude protein retention (CPR, %) was calculated as the gain of whole body protein divided by the dietary crude protein intake during the experiment according to:

$$CPR = (W(t_i) \times BP(t_i)) - (W(t_0) \times BP(t_0)) / (\text{feed consumed } (t_i - t_0) \times DP),$$

where $W(t_i)$ and $W(t_0)$ are the biomass at the end (t_i) and at the beginning (t_0) of the study, respectively, $BP(t_i)$ and $BP(t_0)$ are the body protein content (%) at the end (t_i) and at the beginning (t_0) of the study, respectively, and DP is the dietary protein content.

Apparent crude lipid retention (aCLR, %) was calculated as the gain of whole body lipid divided by the dietary crude lipid intake during the experiment according to:

$$aCLR = (W(t_i) \times BL(t_i)) - (W(t_0) \times BL(t_0)) / (\text{feed consumed } (t_i - t_0) \times DL),$$

where $BL(t_i)$ and $BL(t_0)$ are the body lipid content (%) at the end and at the beginning of the experiment, respectively, and DL is the dietary lipid content (%).

Apparent retention of SAFAs, MUFAs, (n-3) PUFAs, (n-6) PUFAs and total PUFAs were calculated as:

$$a(FA_x)R = (W(t_i) \times BL(t_i) \times BFA_{total}(t_i) \times BFA_x(t_i)) - (W(t_0) \times BL(t_0) \times BFA_{total}(t_0) \times BFA_x(t_0)) / (\text{feed consumed } (t_i - t_0) \times DL \times DFA_{total} \times DFA_x),$$

where x is the fatty acid(s) in question, $BFA_{total}(t_i)$ and $BFA_{total}(t_0)$ are the total fatty acid content of body lipid (in % of BL) at the end and beginning of the study, respectively, $BFA_x(t_i)$ and $BFA_x(t_0)$ are the body content of fatty acid x (in % of BFA_{total}) at the end and at the beginning of the study, respectively, DFA_{total} is the dietary total fatty acid content (in % of DL), and DFA_x is the dietary content of fatty acid x (in % of DFA_{total}).

3.6 Statistical analysis

Data on SGR, FCR, HSI, VSI, CPR, aCLR, proximate and fatty acid compositions of whole fish and fatty acid retention data were subjected to one-way ANOVA using the SigmaStat v3.5 software package. Significant differences were subsequently assessed using the Holm-Sidak all pairwise multiple comparison *post hoc* test. A probability of $P < 0.05$ was considered significant in all analyses.

4. Results

4.1 Diets

The proximate composition of the experimental diets is given in table 1. The analysed starch content increased from 52 g kg⁻¹ in diet A to 230 g kg⁻¹ in diet D, the crude protein content increased slightly from 429 to 456 g kg⁻¹ in the diets, while the lipid content decreased from 228 g kg⁻¹ in diet A to 151 g kg⁻¹ in diet D. The calculated crude

nutrient energy content (CNE) increased from 20.1 MJ kg⁻¹ in diet A to 20.7 MJ kg⁻¹ in diet D. The fatty acid compositions of the four experimental diets were very similar comprising 27.7 – 28.1% SAFAs, 21.5 – 21.6% MUFAs, and 37.1-37.5% PUFAs, while the (n-3)/(n-6) ratio decreased slightly with increasing dietary starch content (Table 2). Between 12.9 – 13.4% of the dietary fatty acids could not be identified on the GC chromatogram.

4.2 Feed intake, growth and feed conversion ratio

Results on FI, FCR and SGR are presented in table 3. The fish accepted all diets well, and total feed waste was negligible (less than 0.01% of feed administered). FCRs ranged between 1.00 - 1.03 with no differences (P=0.064) between dietary treatments. SGRs ranged between 2.61 - 2.66% d⁻¹, decreasing slightly but significantly (P=0.041) with increasing dietary starch content. A subsequent *post hoc* test, however, was unable to identify which dietary treatment groups were significantly different from each other.

4.3 HSI, VSI and proximate composition of whole fish

As presented in table 4, the HSI ranged between 1.53 - 2.22%, increasing significantly (P<0.001) with dietary starch content from diet A, to diet B, to diet C-D. The VSI ranged between 7.85 - 8.56%, with no differences (P>0.05) observed among dietary treatments. No effects of dietary treatment were observed with respect to whole body moisture, crude protein, crude lipid and ash (P>0.05). However, the whole fish glycogen content was lower (P<0.001) in fish fed diets A and B (1.6 and 2.2 g kg⁻¹, respectively) than in fish fed diet C and D (3.1 and 3.8 g kg⁻¹, respectively). Fish displayed a higher moisture content and lower lipid and glycogen content initially than by the end of the study, while whole body crude protein content appeared unchanged.

4.4 Fatty acid composition of whole fish

The initial and final FA compositions of whole fish are presented in table 5. The percentage of whole body FAs comprised by SAFAs and MUFAs at the end of the study was directly related to dietary starch content (and inversely related to dietary lipid content), increasing from 29.4 – 31.6% and 29.7 – 33.5% in fish fed diet A and D, respectively. The percentage of whole body FAs comprised by PUFAs was inversely related to dietary starch content, decreasing from 29.8 – 24.3% in fish fed diet A and D, respectively. The ratio (n-3)/(n-6) decreased significantly with increasing dietary starch level (from 9.1 to 7.6 in diets A and D, respectively). Between 9.3 – 10.9% of the dietary fatty acids could not be identified.

4.5 Crude protein retention (CPR) and apparent crude lipid retention (aCLR)

The crude protein retention (CPR) ranged from 36.4% to 39.1% and was significantly higher in fish fed diet A than fish fed diet D, while the CPR of fish fed diet B or C did not differ significantly from any of the dietary treatment groups. The apparent crude lipid retention (aCLR) increased from 72.8% in fish fed diet A to 102.5% in fish fed diet D, and was significantly higher in fish fed diets C and D than in fish fed diets A and B (Figure 1).

4.6 Apparent retention of SAFAs, MUFAs and PUFAs

The apparent retention (in % of consumed FA) of the major FA groups (SAFAs, MUFAs and PUFAs) is presented in figure 2. The apparent retention of SAFAs increased from 73.3% in fish fed diet A to 118.9% in fish fed diet D, all dietary treatment groups being significantly different from each other ($P < 0.001$). Similarly, the apparent retention of MUFAs increased from 98.6% in fish fed diet A to 164.0% in fish

fed diet D, and all dietary treatment groups differed significantly from each other ($P < 0.001$). The apparent retention of (n-3) PUFAs, (n-6) PUFAs, and total PUFAs varied in the range 56.1 – 61.1%, 68.5 – 80.4% and 57.0 – 62.4%, respectively, and neither of them were significantly affected by dietary treatment ($P > 0.05$).

5. Discussion

A simple, high quality raw material matrix was chosen to assure the highest possible quality of dietary nutrients supplied, and to minimize anti-nutritional effects from compounds such as certain non-starch polysaccharides, protease inhibitors or lectins (Gatlin III. et al., 2007). No negative effects of dietary cellulose on performance and nutrient digestibility have been reported for other species such as European seabass (*Dicentrarchus labrax*), rainbow trout (*Onchorynchus mykiss*) or Atlantic cod (*Gadus morhua*) using inclusion levels up to 200 g kg⁻¹ (Dias et al., 1998; Hansen and Storebakken, 2007; Lekva et al., 2010), thus it was assumed that cellulose could be considered an inert ‘filler’ when balancing dietary energy levels.

The study demonstrated that up to 23% dietary starch could successfully replace lipid as a source of dietary energy without causing adverse effects on feed conversion ratio, proximate composition, or VSI of juvenile gilthead sea bream, while a small, but significant reduction in SGR was observed with increasing dietary starch levels ($p = 0.041$). Both FCRs and SGRs obtained in the present study were, however, good and comparable to those obtained in previous studies on similar sized gilthead sea bream (Lupatsch et al., 2001; Lupatsch et al., 2003; Venou et al., 2003; Couto et al., 2008; Enes et al., 2008a; Enes et al., 2008b), which could most likely be attributed to high energy diets, well-balanced protein-to-energy ratio and EAA profile and a high daily FI.

In accordance to other studies on this (Enes et al., 2009; Enes et al., 2011) and other species (Hemre et al., 2002), whole body glycogen levels and HSIs were significantly elevated with increasing dietary starch level, which is most likely a consequence of excellent starch digestion (Venou et al., 2003; Enes et al., 2008a; Adamidou et al., 2009), combined with an apparent lack of gluconeogenesis regulation by dietary carbohydrates (Meton et al., 1999; Caseras et al., 2002; Enes et al., 2008a; Enes et al., 2008b; Enes et al., 2008c), which in turn have continuously diverted non-carbohydrate metabolites of the glycolytic pathway in the direction of glycogenesis, causing an accumulation of whole body glycogen levels and hepatomegaly in fish fed high starch diets.

Crude protein retention was negatively related to the dietary starch level, indicating that a larger fraction of dietary protein was either fully oxidized for energy purposes when including more starch (and less lipid) in the diet, or may have been deaminated and converted into acetyl-CoA (serving as carbon donor for endogenous lipid biosynthesis) in fish fed high starch diets (figure 1). Another plausible explanation could be that dietary protein levels were slightly elevated with increasing starch inclusion levels, which in turn have increased the dietary protein-to-energy levels, which may have caused elevated protein catabolism in high starch diets. In contrast to CPR, the aCLR was positively related to dietary starch content, and exceeded 100% in fish receiving diet D. These findings substantiate that biosynthesis of lipids *de novo* occurred at least in fish receiving the high starch diets (figure 1), and possibly also in fish fed lower starch diets, when considering the apparent retention of SAFAs and MUFAs. The apparent retention of SAFAs was thus generally positively related to dietary starch level, exceeding 100% in fish fed diets C (105.6%) and D (118.9%), and with 16:0 and

18:0 showing particularly high retention (132.2 and 142.6%, respectively), in fish fed diet D. Consistent with this, the overall content of SAFA (expressed as % of the total FA content) increased from 29.4% in fish fed diet A to 31.6% in fish fed diet D.

The apparent retention of MUFAs was also strongly related to dietary starch level (increasing from 98.6 to 164.0% in fish fed diet A and D, respectively). This response was caused primarily by a very high apparent retention of 18:1(n-9) in all dietary treatment groups (increasing from 116.3% in fish fed diet A to 253.6% in fish fed diet D). The general increase in apparent MUFA retention with increasing dietary starch levels was also reflected in the overall MUFA content of the fish by the end of the trial (increasing from 29.7 to 33.5% in fish fed diet A and D, respectively). Interestingly, this increment was driven solely by 18:1(n-9), while other MUFAs were either not affected by dietary treatment or, as in the case of 16:1(n-7), was negatively correlated to the dietary starch content.

All together, the high apparent retention of SAFAs and MUFAs suggests that: 1) endogenous biosynthesis of new lipids was active and under apparent nutritional control, increasing with increasing dietary starch level and decreasing dietary lipid level; and 2) the main metabolic products of *de novo* lipogenesis (16:0 and 18:0) were readily de-saturated and subsequently elongated to yield 16:1(n-7) and 18:1(n-7), or 18:1(n-9), 20:1(n-9), 22:1(n-9), and 24:1(n-9), respectively.

In comparison to SAFAs and MUFAs, the apparent retention of total PUFAs, (n-3) PUFAs and (n-6) PUFAs was not significantly affected by dietary treatment. As a consequence, fish fed increasing dietary starch levels (and thus decreasing dietary lipid levels) showed a significant decrease in the content of (n-3) PUFAs, (n-6) PUFAs and total PUFAs. Thus, by the end of the study, the PUFA content of the fish more or less

reflected the dietary contribution of those FAs. These results do not come as a surprise considering that the two major contributors to that group, 20:5(n-3) (EPA) and 22:6(n-3) (DHA) could be considered practically essential due to the very low fatty acyl $\Delta 5$ desaturase activity found in this species (Tocher and Ghioni, 1999), rendering further desaturation of 20:4(n-3) to EPA and DHA insignificant.

Interestingly, the apparent retention of 22:5(n-3) (DPA) exceeded 100% irrespective of dietary treatment, suggesting that some DPA may have originated from the elongation of EPA rather than just from the diet. Similar results have been reported previously in both this (Mourente and Tocher, 1994) and other marine fish species (Takeuchi et al., 1996; Bell et al., 1995).

In conclusion, this study showed that while growth, feed performance and proximate composition of juvenile gilthead sea bream were virtually all un-affected by shifting the dietary energy source from lipid to starch, both FA retention dynamics and final FA profile of the fish were clearly influenced by the shift. The apparent retention of SAFAs and MUFAs were positively related to dietary starch level (and negatively related to dietary lipid level), exceeding 100% in fish fed high starch diets. These findings substantiate that considerable *de novo* lipogenesis was taking place and apparently subject to nutritional control, while apparent retention of PUFAs appeared to be unaffected by dietary treatment. Combined, this caused the SAFA and MUFA content of the fish to increase and the PUFA content to decrease, adversely affecting the overall FA quality of the final product.

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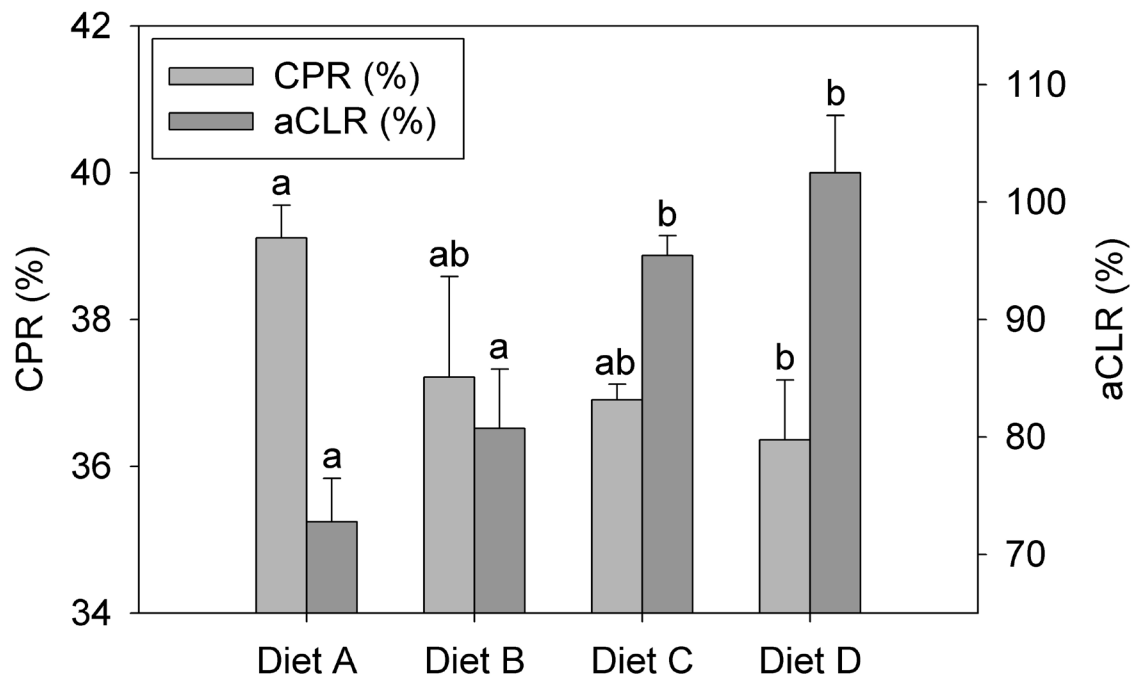


Figure 1. The crude protein retention (CPR) and apparent crude lipid retention (aCLR) of gilthead sea bream (mean \pm STD, $n=3$) fed four diets differing in dietary starch content (A=52 g kg⁻¹; B=119 g kg⁻¹; C=175 g kg⁻¹; D=230 g kg⁻¹) for a period of 41 days. One way ANOVA showed that both CPR and aDLR were significantly affected by dietary treatment ($p=0.018$ and $p<0.001$, respectively), while different letters above bars indicate significant differences among dietary treatments found by the subsequent *post hoc* test (Holm-Sidak, $p<0.05$).

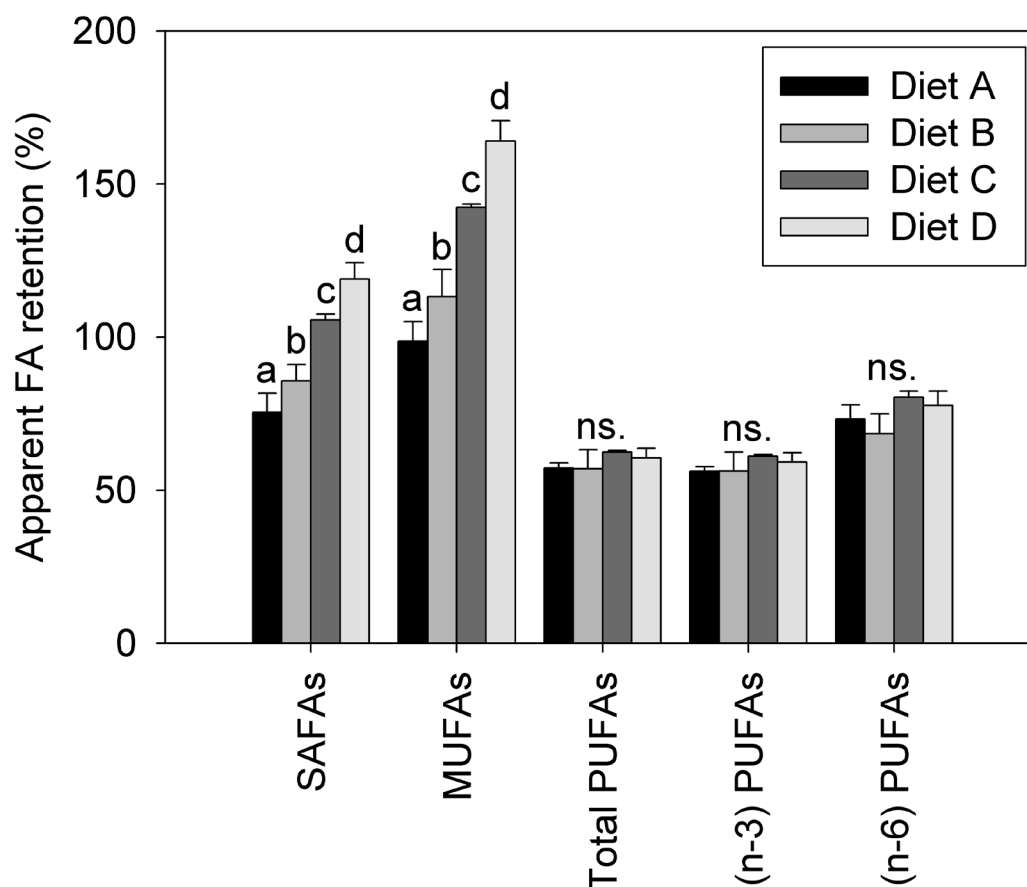


Figure 2. The apparent fatty acid retention (%) of gilthead sea bream (mean \pm STD, $n=3$) fed four diets differing in dietary starch content (A=52 g kg⁻¹; B=119 g kg⁻¹; C=175 g kg⁻¹; D=230 g kg⁻¹) for a period of 41 days. One way ANOVA showed that apparent retention of both SAFAs and MUFAs were significantly affected by dietary treatment ($p<0.001$), while apparent retention of total PUFAs ($p=0.259$), (n-3) PUFAs ($p=0.308$) and (n-6) PUFAs ($p=0.063$) were not significantly affected by dietary treatment. Different letters above bars indicate significant differences among dietary treatments found by the subsequent *post hoc* test (Holm-Sidak, $p<0.05$).

9. Tables

Table 1 Diet formulation and proximate composition (g kg^{-1}) of diets containing increasing levels of wheat starch and decreasing levels of fish oil.

Diet	A	B	C	D
<i>Ingredients (g kg^{-1})</i>				
Fish meal, Con-kix ¹	607	607	607	607
Fish oil, Peru	152	127	103	78
Wheat starch ²	68	137	205	274
Cellulose ³	159	115	71	27
Guar gum ⁴	5	5	5	5
Vitamin and mineral premix ⁵	3	3	3	3
Monocalcium phosphate	6	6	6	6
<i>Proximate composition (g kg^{-1})</i>				
Crude protein	429	433	445	456
Crude fat	228	201	178	151
Starch	52	119	175	230
Ash	75	75	77	79
Water	68	65	65	74
Phosphorus	14	14	15	15
Crude nutrient energy (CNE, MJ kg^{-1}) ⁶	20.1	20.3	20.6	20.7

¹TripleNine Fish Protein, Esbjerg, Denmark.

²Cargill, Belgium.

³Vitacel R200, Rettenmaier und Söhne GmbH, Germany.

⁴Guar gum HV200, LCH A/S, Frederiksberg, Denmark.

⁵Vitamins and minerals (kg feed⁻¹): vitamin A 3750 IU, cholecalciferol (D₃) 750 IU, α-tocopherol (E) 131.3 mg, thiamine 7.5 mg, riboflavin 15 mg, pyridoxine 7.5 mg, vitamin B₁₂ 2.25 µg, vitamin K₃ 7.5 mg, zinc 75 mg, iodine 0.9 mg, copper 3.75 mg, manganese 22.5 mg, cobalt 0.75 mg, selenium 0.19 mg.

⁶Crude nutrient energy was calculated as the sum of the dietary content of protein, lipid and starch, multiplied by their respective energetic values upon complete oxidation (Blaxter. 1989):

$$\text{CNE (MJ/kg)} = (\text{P}_{\text{diet}} \times 23.7) + (\text{L}_{\text{diet}} \times 39.6) + (\text{S}_{\text{diet}} \times 17.2)$$
, where P_{diet} , L_{diet} and S_{diet} are the dietary protein, lipid and starch contents (%), respectively.

535 **Table 2** Analysed fatty acid composition (% of total fatty acids) of the experimental
536 diets.

Diet	A	B	C	D
14:0	7.1	7.0	6.8	6.6
16:0	17.0	17.2	17.1	17.1
18:0	2.8	2.9	2.8	2.9
Total saturates ¹	28.1	28.2	27.9	27.7
16:1(n-7)	7.0	7.0	6.7	6.5
18:1(n-9)	7.7	7.6	7.6	7.4
18:1(n-7)	2.2	2.2	2.2	2.1
20:1 ²	3.7	3.9	4.1	4.5
22:1(n-9)	0.3	0.3	0.4	0.4
24:1(n-9)	0.5	0.5	0.6	0.7
Total monoenes	21.6	21.5	21.5	21.5
18:2(n-6)	1.4	1.5	1.5	1.8
20:2(n-6)	0.2	0.2	0.2	0.2
22:5(n-6)	0.3	0.3	0.3	0.3
Total (n-6) ³	2.3	2.4	2.5	2.7
18:3(n-3)	1.0	1.1	1.1	1.1
18:4(n-3)	3.5	3.5	3.5	3.5
20:3(n-3)	0.7	0.7	0.7	0.7
20:5(n-3); EPA	14.7	14.5	14.1	13.8
22:5(n-3); DPA	1.8	1.8	1.7	1.6
22:6(n-3); DHA	13.2	13.5	13.6	14.1

Total (n-3)	34.9	35.0	34.6	34.8
(n-3)/(n-6) PUFA	15.4	14.8	14.1	12.9
Total PUFA	37.1	37.4	37.1	37.5
Unidentified fatty acids ⁴	13.2	12.9	13.4	13.2

537 ¹Includes also 15:0, 17:0, 20:0 and 22:0.

538 ²Includes also 20:1(n-11) and 20:1(n-9).

539 ³Includes also 18:3(n-6), 20:3(n-6), 20:4(n-6) and 22:4(n-6).

540 ⁴Comprising fatty acids not recognized on the GC chromatogram.

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Table 3 Fish biomass, growth rates (SGR), feed intake (FI), and feed conversion ratios (FCR) (mean \pm STD, n=3)¹ of fish fed the experimental diets

Diet	A	B	C	D	p-value
<i>Biomass, individual weights and feed intake (g)</i>					
Initial biomass	2027 \pm 8	2018 \pm 8	2003 \pm 3	2015 \pm 5	-
End biomass	5941 \pm 58	5849 \pm 27	5728 \pm 73	5787 \pm 86	-
Init. ind. weight	25.3 \pm 0.1	25.2 \pm 0.1	25.0 \pm 0.0	25.2 \pm 0.1	-
End ind. weight	74.6 \pm 0.2	73.1 \pm 0.3	72.2 \pm 0.8	72.6 \pm 0.6	-
FI	3916.3 \pm 22.3	3900.8 \pm 4.6	3855.6 \pm 4.4	3892.8 \pm 2.5	-
<i>Feed conversion and specific growth rate</i>					
FCR (kg kg ⁻¹) ²	1.00 \pm 0.01	1.02 \pm 0.00	1.03 \pm 0.01	1.03 \pm 0.02	0.064
SGR (% day ⁻¹) ^{3,4}	2.66 \pm 0.02	2.63 \pm 0.01	2.61 \pm 0.03	2.61 \pm 0.02	0.041

¹Values within a row not sharing the same superscript letter were significantly different (Holm-Sidak, P<0.05).

² FCR = feed consumed / biomass gain.

³ SGR = $\ln(\text{biomass}_{\text{end}} / \text{biomass}_{\text{initial}}) / (\text{days in trial}) \times 100$ (Hopkins, 1992).

⁴ ANOVA analyses found significant difference between treatment groups. However, the subsequent *post hoc* test (Holm-Sidak) was unable to identify which dietary treatment groups were significantly different from each other.

Table 4 Chemical composition of whole fish and hepatosomatic and viscerosomatic indices (mean \pm STD, n=3)¹

	Initial	Diet A	Diet B	Diet C	Diet D	p-value
fish						
<i>Chemical composition of whole fish (g kg⁻¹)</i>						
Moisture	707 \pm 1	661 \pm 6	663 \pm 7	656 \pm 2	659 \pm 5	0.520
Crude protein	166 \pm 2	167 \pm 1	165 \pm 3	168 \pm 1	169 \pm 1	0.149
Crude lipid	99 \pm 0	143 \pm 6	143 \pm 7	148 \pm 3	138 \pm 6	0.271
Glycogen	0.6 \pm 0.0	1.6 \pm 0.3 ^a	2.2 \pm 0.5 ^a	3.1 \pm 0.5 ^b	3.8 \pm 0.3 ^b	<0.001
Ash	39 \pm 1	35 \pm 1	34 \pm 1	34 \pm 1	37 \pm 2	0.221
<i>Hepatosomatic and viscerosomatic indices (%)</i>						
HSI ²	n/a	1.53 \pm 0.16 ^a	1.88 \pm 0.28 ^b	2.22 \pm 0.34 ^c	2.15 \pm 0.41 ^c	<0.001
VSI ³	n/a	8.37 \pm 1.09	7.85 \pm 0.90	8.56 \pm 0.71	8.49 \pm 0.94	0.059

¹Values within a row not sharing the same superscript letter were significantly different (Holm-Sidak, P<0.05), excluding initial fish from the analysis.

²HSI = (weight_(liver) / weight_(whole body)) \times 100.

³VSI = (weight_(viscera) / weight_(whole body)) \times 100.

Table 5 Analysed fatty acid composition (% of total fatty acids) and apparent retention (%) of individual fatty acids (in brackets) for initial fish and fish fed four experimental diets for a period of 41 days (mean \pm STD, n=3)¹

	Start fish	Diet A	Diet B	Diet C	Diet D	p-value
14:0	6.3	6.6 \pm 0.1 ^a (67.9 \pm 5.1) ^a	6.3 \pm 0.1 ^b (71.0 \pm 4.1) ^{ab}	5.9 \pm 0.1 ^c (79.7 \pm 1.7) ^{bc}	5.5 \pm 0.0 ^d (81.9 \pm 3.9) ^c	<0.001 (0.006)
16:0	18.9	18.9 \pm 0.5 ^a (79.6 \pm 6.8) ^a	19.7 \pm 0.3 ^b (91.6 \pm 6.5) ^b	20.5 \pm 0.2 ^c (115.2 \pm 1.5) ^c	21.4 \pm 0.0 ^d (132.2 \pm 5.8) ^d	<0.001 (<0.001)
18:0	3.7	3.1 \pm 0.1 ^a (73.2 \pm 6.9) ^a	3.5 \pm 0.2 ^b (94.2 \pm 3.5) ^b	3.7 \pm 0.1 ^c (121.9 \pm 4.6) ^c	3.9 \pm 0.0 ^c (142.6 \pm 7.6) ^d	<0.001 (<0.001)
Total saturates ²	30.1	29.4 \pm 0.8 ^a (75.3 \pm 6.3) ^a	30.4 \pm 0.6 ^{ab} (85.6 \pm 5.4) ^b	31.0 \pm 0.3 ^b (105.6 \pm 1.9) ^c	31.6 \pm 0.1 ^b (118.9 \pm 5.4) ^d	0.005 (<0.001)
16:1(n-7)	9.0	9.0 \pm 0.2 ^a (92.3 \pm 6.6) ^a	8.7 \pm 0.1 ^b (97.3 \pm 7.2) ^a	8.4 \pm 0.1 ^c (115.1 \pm 1.4) ^b	8.2 \pm 0.0 ^d (125.1 \pm 6.0) ^b	<0.001 (<0.001)
18:1(n-9)	14.7	12.9 \pm 0.2 ^a (116.3 \pm 7.4) ^a	14.2 \pm 0.2 ^b (146.1 \pm 12.9) ^b	15.8 \pm 0.1 ^c (199.8 \pm 2.1) ^c	17.4 \pm 0.1 ^d (253.6 \pm 11.9) ^d	<0.001 (<0.001)
18:1(n-7)	3.9	3.2 \pm 0.1 (96.8 \pm 7.4) ^a	3.2 \pm 0.1 (106.7 \pm 6.9) ^a	3.2 \pm 0.0 (126.5 \pm 1.5) ^b	3.1 \pm 0.0 (137.8 \pm 4.8) ^b	0.088 (<0.001)
20:1 ³	1.8	3.4 \pm 0.1	3.5 \pm 0.1	3.5 \pm 0.0	3.5 \pm 0.1	0.114

22:1(n-9)	0.3	(74.6±3.9) ^a (114.6±9.8) ^a	(80.6±4.5) ^a (141.4±5.9) ^b	(91.6±0.9) ^b (152.4±2.0) ^{bc}	(92.5±2.0) ^b (156.7±2.3) ^c	(<0.001) 0.041 (<0.001)
24:1(n-9)	0.5	0.6±0.1 (91.4±12.8) ^a	0.6±0.0 (104.0±14.5) ^{ab}	0.7±0.0 (122.5±7.4) ^b	0.7±0.0 (119.6±3.4) ^{ab}	0.182 (0.022)
Total monoenes	30.2	29.7±0.4 ^a (98.6±6.4) ^a	30.7±0.3 ^b (113.3±8.8) ^b	32.1±0.1 ^c (142.4±1.1) ^c	33.5±0.0 ^d (164.0±6.7) ^d	<0.001 (<0.001)
18:2(n-6)	4.3	2.1±0.0 (77.5±7.6)	2.1±0.0 (74.6±8.3)	2.1±0.0 (87.8±1.5)	2.1±0.0 (81.2±5.4)	0.065 (0.135)
20:2(n-6)	0.2	0.2±0.0 (69.1±12.0) ^a	0.2±0.0 (73.5±6.9) ^{ab}	0.2±0.0 (89.9±3.5) ^b	0.2±0.0 (88.7±1.5) ^{ab}	0.777 (0.016)
22:5(n-6)	0.3	0.3±0.1 ^a (97.1±16.8) ^a	0.2±0.0 ^b (53.1±4.6) ^b	0.2±0.0 ^b (62.9±5.3) ^b	0.2±0.0 ^b (66.9±3.3) ^b	0.002 (0.002)
Total (n-6) ⁴	5.1	3.0±0.0 ^a (73.2±4.6)	2.8±0.1 ^b (68.5±6.4)	2.8±0.0 ^b (80.4±2.0)	2.8±0.0 ^b (77.6±4.7)	0.001 (0.063)
18:3(n-3)	0.8	0.9±0.0 ^a (67.1±3.5)	0.9±0.0 ^b (67.5±7.0)	0.8±0.0 ^b (75.1±0.7)	0.8±0.0 ^c (73.9±4.2)	<0.001 (0.119)

18:4(n-3)	1.6	2.5±0.0 ^a (57.9±1.9)	2.3±0.1 ^b (58.0±6.7)	2.2±0.0 ^c (62.6±1.3)	2.0±0.0 ^d (61.6±2.5)	<0.001 (0.352)
20:3(n-3)	0.8	0.6±0.0 ^a (54.6±2.7)	0.6±0.0 ^b (53.9±5.2)	0.5±0.0 ^c (58.5±1.2)	0.5±0.0 ^d (58.7±1.6)	<0.001 (0.194)
20:5(n-3); EPA	10.5	10.3±0.2 ^a (50.1±1.5)	9.5±0.3 ^b (50.2±6.2)	8.7±0.1 ^c (54.3±0.7)	7.9±0.1 ^d (52.5±3.1)	<0.001 (0.459)
22:5(n-3); DPA	3.0	2.7±0.0 ^a (108.2±5.5)	2.6±0.1 ^b (107.3±10.9)	2.4±0.0 ^c (124.2±1.3)	2.2±0.0 ^d (120.7±5.6)	<0.001 (0.033)
22:6(n-3); DHA	8.7	9.8±0.3 ^a (54.7±1.0)	9.2±0.2 ^b (54.6±5.4)	8.6±0.1 ^c (59.1±0.5)	8.1±0.0 ^d (56.7±2.7)	<0.001 (0.305)
Total (n-3)	25.4	26.8±0.5 ^a (56.1±1.5)	25.0±0.5 ^b (56.2±6.2)	23.2±0.2 ^c (61.1±0.6)	21.5±0.1 ^d (59.2±3.0)	<0.001 (0.308)
Total PUFA	30.5	29.8±0.5 ^a (57.2±1.7)	27.8±0.5 ^b (57.0±6.2)	26.0±0.2 ^c (62.4±0.6)	24.3±0.1 ^d (60.5±3.1)	<0.001 (0.259)
(n-3)/(n-6) PUFA	5.0	9.1±0.2 ^a	9.0±0.3 ^a	8.3±0.1 ^b	7.6±0.0 ^c	<0.001
Unidentified fatty acids ⁵	8.92	10.9±0.9	10.8±0.5	9.3±0.3	10.0±0.7	0.055

¹Values within a row not sharing the same superscript letter were significantly different (Holm-Sidak, P<0.05).

561 ²Includes also 15:0, 17:0, 20:0 and 22:0. ³Includes also 20:1(n-11) and 20:1(n-9). ⁴Includes also 18:3(n-6), 20:3(n-6), 20:4(n-6) and 22:4(n-

562 6). ⁵Comprising fatty acids not recognized on the GC chromatogram.

5. Short summary of main results and discussion

The main objective of the work behind the present thesis was to assess the effects of dietary nutrient composition on *de novo* lipogenesis in cultured gilthead sea bream. Additionally, dietary effects on glycogenesis, growth and feed performance, body composition and nutrient retention efficiencies were also assessed. All experimental fish were obtained from Ferme Marine de Douhet, Île d'Oléron, France and all biological trials were carried out at BioMar's feed trial unit in Hirtshals, Denmark.

In paper I the metabolic fate of dietary starch was assessed using four iso-DP and iso-DE diets where the dietary starch level was gradually increased from 6 to 24% at the expense of dietary lipid (fish oil). Using trace amounts of ^{13}C labelled dietary starch, the temporal ^{13}C enrichment of hepatic and whole fish glycogen and lipid pools was measured, allowing quantification of glycogenesis and *de novo* lipogenesis.

Quantification of *de novo* lipogenesis of protein origin, and the importance of this in the overall whole body lipid budget was determined in paper II using nine diets comprising 3 DP levels (33, 36 and 40%) and 3 DE levels (19.5, 20.5 and 21.5 MJ/kg). The addition of trace amounts of ^{13}C labelled dietary protein allowed measurement of ^{13}C enrichment of the whole body lipid, enabling quantification *de novo* lipogenesis of protein origin.

Paper III describes the consequences of increasing dietary starch levels on whole body protein retention, apparent lipid and fatty acid retention efficiencies, and fatty acid composition of the fish using four diets similar to the ones used in paper I, only without the addition of stable isotopes.

5.1 *De novo* lipogenesis

The results from paper I and II confirm that gilthead sea bream is indeed capable of synthesising lipid *de novo* from both dietary starch and protein sources. In paper I, fish fed increasing dietary starch levels (from 6 to 24%) showed starch ADCs values between 96.5 and 99.3%, which corroborates the very high ADCs of processed/extruded starches obtained earlier by several other authors^(13, 18, 64, 77). Between 4.2 to 8.4% of the starch digested was converted to lipid *de novo*, corresponding to deposition rates between 18.7 to 123.7 mg/kg biomass/day. Additionally, a direct relation was found between starch intake and lipid deposition of starch origin. This agrees well with earlier studies showing an up-regulation of lipogenic enzyme activity (glucose-6-phosphate dehydrogenase (G6PD)) with increasing dietary carbohydrate intake^(13, 22, 26, 72, 94). In turn, this implies that that fish, irrespective of

dietary treatment, used more than 90% of the digested starch for purposes other than lipogenesis, which was also reflected in the modest contribution of lipid synthesised from starch *de novo* to total lipid deposition; less than 9% of total lipid deposition during the trial behind paper I could be attributed to digested starch. Conversely, a much larger fraction of the digested protein appear to contribute to *de novo* lipogenesis in gilthead sea bream. In the study behind paper II, fish were fed nine diets differing in dietary DP/DE ratio and energy density. This study showed that between 18.6 and 22.4% of the digested protein was converted to lipid *de novo*. Consequently, lipid of protein origin comprised between 21.6 and 30.3% of the total whole body lipid deposited throughout the trial. Thus, over a wide range of dietary DP/DE ratios, including ratios considerably lower than previously recommended for gilthead sea bream^(53, 54), the fish appears to consequently convert approximately one-fifth of the DP to lipid *de novo*. Figueiredo-Silva⁽⁹⁵⁾ similarly found that *de novo* lipogenesis in blackspot seabream (*Pagellus bogaraveo*) was more related to dietary protein level than starch. In rainbow trout juveniles (*Onchorynchus mykiss*), Campbell⁽³²⁾ found between 9.7 and 44.5% of whole body lipids could be attributed to dietary protein. In addition, contrary to the present study, the fraction of protein converted into lipids was strongly related to the dietary DP/DE level in that study. In paper III, fish were fed diets similar to those used in paper I, except without the addition of ¹³C labelled starch. Thus, no direct proof of active *de novo* lipogenesis was apparent in fish in this trial. However, considering that the amount of crude lipid, SAFAs and MUFAs accreted by fish fed high starch diets during the trial exceeded what was offered through the diet, the results strongly suggest that *de novo* lipogenesis was indeed active. Particularly 16:0 (palmitic acid) and 18:0 (stearic acid) fatty acids, which are considered to be the primary products of *de novo* lipogenesis⁽⁸⁹⁾, and their desaturated successor 18:1n-7, displayed very high accretion levels in fish fed high starch diets (132, 143 and 254% of the amount offered through the diet, respectively).

5.2 Glycogenesis

Besides the impressive starch ADC values already mentioned in section 1.6.1, results from paper I clearly demonstrated that endogenous glycogen synthesis of starch origin was generally active, and highly stimulated by increasing dietary starch content. Thus, glycogen of starch origin comprised between 27.2 (diet A; 6% starch) and 68.8% (diet D; 24% starch) of the total hepatic glycogen synthesis, while between 16.5 (diet A; 6% starch) and 38.8% (diet D; 24% starch) of the whole body glycogen pool could be attributed to dietary starch. Also, the turnover of both hepatic and whole body glycogen pools was clearly elevated by

increasing dietary starch. These results support the general perception that both starch digestion^(13, 18, 64, 77) and the following liver glucose phosphorylation^(18-20, 25, 27) (glucose → glucose-6-phosphate (G6P)) are very efficient in gilthead sea bream, even at high dietary starch inclusion levels. The dietary starch contribution to hepatic and whole body glycogen pools implies that almost two thirds of the whole body glycogen and approximately one third of the liver glycogen must have originated from sources other than dietary starch, even when feeding the high starch diet (diet D; 24% starch). This could potentially be attributed to lack of dietary regulation of FbPase^(18-20, 22, 25), continuously diverting non-carbohydrate metabolites of the glycolytic pathway in the direction of glycogenesis. The combined endogenic and dietary contribution to glycogenesis appeared to have caused an accumulation of glycogen in both whole fish and liver tissues causing hepatomegaly in fish fed high starch diets^(11, 13, 79).

5.3 Growth, feed utilization and feed intake

The feeding strategy applied in all three trials behind the supporting papers was restricted iso-energy feeding. The main reason for choosing a sub-maximal feeding strategy was an observed crusting of feed pellets when fish were fed to near satiation⁽⁹⁶⁻⁹⁸⁾, rendering accurate quantification of wasted feed pellets impossible. This, in turn, would have had adverse effects on assessment of feed intake, feed conversion ratios, nutrient retention calculations and isotopic enrichment calculations. Thus, throughout all trials feed intake was adjusted according to iso-energy just below satiation. As a consequence of the tight relation between dietary energy intake and growth (when dietary protein is not a limiting factor) reported earlier^(53, 54), fish growth (SGR) was virtually unaffected by dietary treatment in all three trials. Thus SGR results from paper I, II and III ranged between 1.19 and 1.25% (fish growing from 75-100 grams), 0.96 and 1.05% (fish growing from 140-340 grams) and 2.61 and 2.66% (fish growing from 25-75 grams), respectively. Similarly, no significant effects of dietary treatment on feed conversion ratios were found in paper I and III. This could be explained by the close relation between dietary energy density (DE) and obtained FCR reported earlier both in this^(53, 54) and other species^(58, 65). This relation was corroborated further by the FCR results obtained in paper II (see figure 12), which showed that FCRs were clearly lowered by increasing dietary digestible energy density (DE).

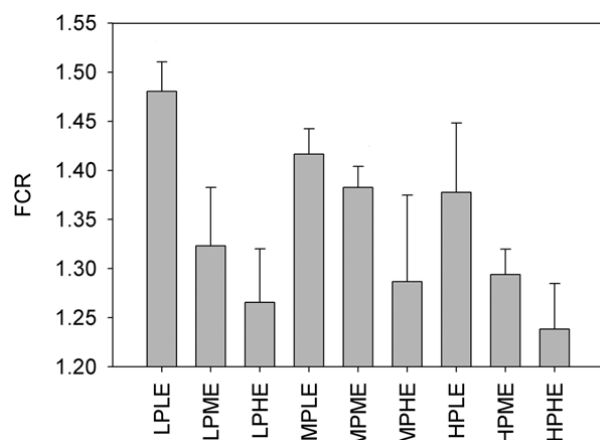


Figure 12. Feed conversion ratios (FCRs) obtained from feeding gilthead sea bream nine diets differing in digestible protein (DP) content (LP=33% DP; MP=36% DP; HP=40% DP) and digestible energy (DE) content (LE=19.5 MJ/kg; ME=20.5 MJ/kg; HE=21.5 MJ/kg).

5.4 Nutrient and energy retention efficiencies

The effects of dietary nutrient composition and energy density on digestible protein retention (DPR), apparent digestible lipid retention (aDLR), and digestible energy retention (DER) were assessed in paper II. The DPR results showed increasing retention efficiencies with decreasing DP and/or increasing DE level (i.e. decreasing DP/DE ratio) (figure 13: A).

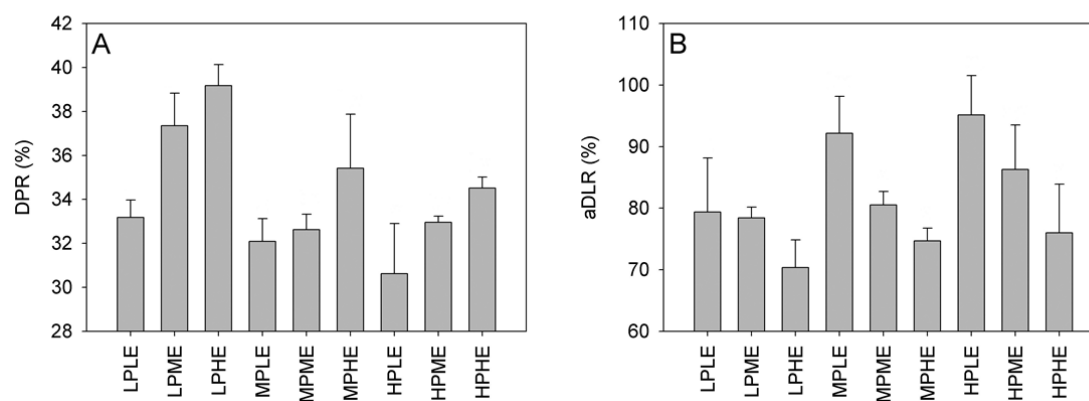


Figure 13. A: Digestible protein retention (DPR) and B: apparent digestible lipid retention (aDLR) of gilthead sea bream fed nine diets differing in digestible protein (DP) content (LP=33% DP; MP=36% DP; HP=40% DP) and digestible energy (DE) content (LE=19.5 MJ/kg; ME=20.5 MJ/kg; HE=21.5 MJ/kg).

These results substantiate the so-called protein sparing effect of substituting DE originating from DP with DE from non-protein sources as already reported from a number of aquacultured species^(58, 99), including gilthead sea bream^(26, 52, 100). Conversely, the highest aDLR values were found in fish fed low energy/high protein diets (figure 13: B). Thus, the

aDLR and DPR results combined suggest that while protein was spared by a decreasing dietary DP/DE level, the opposite was true for lipid, substantiating that deaminated DP was indeed converted into body lipids as indicated by the isotope enrichment results. The result of these opposing nutrient retention dynamics have supposedly rendered differences in the overall digestible energy retentions (DERs) insignificant ($p>0.05$) in that experiment.

Interestingly, in paper III where fish were fed four diets containing increasing amounts of dietary starch, but similar DP and DE contents, a sizeable effect of dietary treatment on apparent crude lipid retention (aCLR) was found (figure 14). The obvious explanation of this direct relation between dietary starch level and aCLR would be *de novo* lipogenesis utilising starch as a carbon donor. However, considering the relatively modest contribution of starch carbon to total lipid deposition found in paper I, the explanation for these dynamics should probably be found elsewhere. Perhaps a larger fraction of the non-retained protein (α -keto acids) was oxidized for energy purposes in fish fed the low starch diets, whereas this energy was covered by glucose (starch) oxidation in fish fed the high starch diets, leaving more of the non-retained protein available for *de novo* lipid synthesis *via* acetyl-CoA. The latter point has not been resolved in the present thesis. However, considering the relatively modest contribution of starch carbon to total lipid deposition, combined with aCLR values in excess of 100%, the overall results strongly hint that a significant portion of the lipid deposited in fish fed the high starch diets originated from *de novo* lipogenesis using protein as the carbon donor.

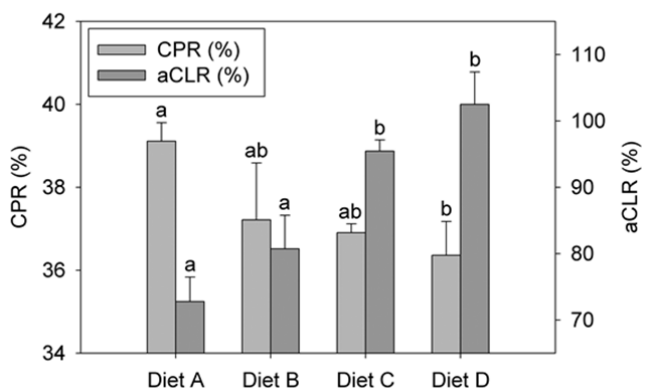


Figure 14. The crude protein retention (CPR) and apparent crude lipid retention (aCLR) of gilthead sea bream (mean \pm STD, $n=3$) fed four diets differing in dietary starch content (A=52 g/kg; B=119 g/kg; C=175 g/kg; D=230 g/kg) for a period of 41 days.

5.5 Chemical composition of whole fish

On average fish trialled in paper I grew from approximately 75 to 110 grams over a feeding period of 30 days, while fish in paper II grew from approximately from 140 to 350 grams over a feeding period of 89 days. Fish trialled in paper III grew from approximately 25 to 75

grams over a feeding period of 41 days. Despite the substantial differences in nutrient and energy content of the experimental diets used in both paper I and III (starch content increasing from 6 to 24%), and between the diets used in paper II (DP ranging between 33 to 40%; DE ranging between 19.5 and 21.5 MJ/kg) no significant differences in final whole body MOPA (moisture, oil, protein and ash) was apparent in any of the three trials. The only exception to this was a small but significant increase whole body protein with increasing dietary protein level found in paper II (increasing from approximately 17.0 to 17.3%). This observation may be surprising considering the relationship between dietary digestible carbohydrate level and resulting whole body lipid level established earlier^(18, 22, 26, 62). Also, a decreasing dietary DP/DE level (increasing DE level) have been known to increase whole body lipid deposition in gilthead sea bream⁽¹⁰¹⁾. In contrast, other studies found body lipid levels to be unaffected by both macro nutrient ratios in iso-DE diets⁽²¹⁾ and dietary energy level⁽¹⁰²⁾.

Considering the final whole body composition of fish from the three trials comprised in the present thesis, it appears as if the main determinant of whole body lipid content is more likely to be the digestible energy intake rather than the dietary nutrient composition or energy content. In addition to MOPA analyses also fatty acid analyses on feed, initial fish and end fish were carried out in paper III. These results showed that an increase in dietary starch content from 6 to 24% lead to a significant increase in whole body SAFA and MUFA contents (increasing from 29.4 to 31.6% and 29.7 to 33.5% of the total fatty acids, respectively), which consequently decreased whole body PUFA content (from 29.8 to 24.3% of the total fatty acids). A likely explanation for these results is a substantial increase in *de novo* lipogenesis in fish fed high starch diets as already described in the last part of section 1.6.1.

6. Conclusions

- *De novo* lipogenesis in gilthead sea bream is active and under apparent nutritional control. Results showed that between 4.2 and 8.4% of digested starch was converted into body lipids *de novo*, corresponding to a synthesis rate of 18.7 to 123.7 mg/kg biomass/day, when fed diets ranging between 6 and 24% dietary starch, respectively. Between 18.6 and 22.4% of the digested protein was converted to lipid *de novo*, corresponding to between 21.6 and 30.3% of the total lipid deposited in fish, using nine experimental diets differing in dietary DP and DE levels.
- Gilthead sea bream efficiently digests processed/extruded starches and up to 68.8% of the hepatic glycogen pool could be attributed to dietary starch, while the same was true for up to 38.8% of the whole body glycogen pool. In turn, this implies that almost two thirds of the whole body glycogen and approximately one third of the liver glycogen must have originated from sources other than dietary starch, even when feeding the high starch diet, substantiating an apparent lack of FbPase regulation by dietary carbohydrates found in other studies. As a consequence, the combined dietary and endogenous contribution to glycogenesis appear to have caused an accumulation of glycogen in both hepatic and whole body tissues causing hepatomegaly in fish fed high starch diets.
- The very clear improvement of FCR with increasing dietary energy level combined with an improvement of digestible protein retention with decreasing DP/DE levels suggest that gilthead sea bream are capable of efficiently utilising feeds within a wide range of dietary DP/DE ratios and energy densities.
- Considering lipogenesis results, nutrient retention efficiencies and body composition results obtained in the three trials collectively, gilthead sea bream appear to endeavour to rigorously maintain a certain whole body energy status under a wide variety of dietary DP/DE ratios, energy densities and nutrient compositions, even if substantial amounts of dietary protein is sacrificed to achieve this. This may indicate that this species has evolved to maximise energy storage in the form of lipid for seasonal, migratory or maturation purposes at the expense of increasing body size through more

efficient use of protein for growth. *De novo* lipogenesis appear to play a key role in maintaining this energy homeostasis.

- Substituting dietary energy supplied from lipid with energy from carbohydrates may inflict a substantial increase in endogenous lipid synthesis (most likely originating from dietary protein), which in turn causes an increase in whole body SAFA and MUFA levels, while PUFA levels decreases.

7. Future perspectives

The fact that dietary (digested) major nutrients are not destined to one particular function in the body complicates the task of optimising diets for farmed animals. As will be described in the following chapters all macro nutrients have the potential of being endogenously converted into other nutrients or metabolites. Thus, despite accurately knowing the nutritional inputs to such an optimisation, interpretation of biological responses should be done cautiously. However, tracing and quantifying the metabolic fate of major nutrients and thereby obtaining a greater insight into the endogenous mechanisms behind these biological responses might enable us to optimise diets more correctly in the future.

Throughout the work of this thesis some interesting questions concerning the metabolic fate of dietary macro nutrients in gilthead sea bream were answered. However, many new questions, which need to be investigated, have also arisen during this work. Some of them are listed below.

- The present thesis found that protein over a wide range of dietary DP/DE ratios and energy densities was extensively deaminated and endogenously converted into body lipids. It was hypothesized that this was the result of an energy conserving strategy in which gilthead sea bream prioritises lipid deposition for migratory, seasonal or maturation purposes, at the expense of increasing body size through more efficient use of protein for growth. However, a similar physiological response would probably be apparent if this species exerted preferential oxidation or endogenous conversion of certain essential amino acids (EAAs), which otherwise was believed (from traditional EAA optimisation) to be available for growth (protein accretion). This, in turn, would render a larger fraction of the overall amino acid pool useless for protein synthesis (because of the lack of an essential amino acid). Consequently, a considerable amount of digested amino acids would be deaminated, leaving the carbon residue (α -keto acid) available for either *de novo* lipogenesis or complete oxidation. Whether or not the above applies could be elegantly tested by feeding a ^{13}C labelled protein tracer and subsequently measure the incorporation of individual ^{13}C labelled EAAs in body protein of the fish, allowing for a determination of ‘true’ retention of individual EAAs using Liquid Chromatography – Isotope Ratio Mass Spectrometry (LC-IRMS).
- If the relatively extensive conversion of digestible protein into body lipids was indeed the results of an intentional energy conserving strategy, one may consider to replace

parts of the dietary protein fraction with 'low quality protein' (i.e. lower demand for amino acid composition), since approximately 20% of the digestible protein fraction appears to be immediately deaminated and converted into lipid *de novo* regardless of dietary protein inclusion level. This would need to be investigated.

- Performance results and dietary effects on whole body composition combined suggest that gilthead sea bream are able to efficiently utilise diets over a wide range of DP:DE ratios, energy densities and nutrient compositions. However, the extensive glycogenesis found in fish fed high dietary starch levels (paper I), causing both increased HSIs and whole body and hepatic glycogen levels, suggest that high dietary starch levels could have adverse effects on general fish health. This would need to be investigated prior to commercial implementation of high dietary starch levels.

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